REMARKS

Favorable reconsideration of this application, in light of the preceding amendments and following remarks, is respectfully requested.

Claims 1-7 and 10 are pending in this application. No claims are amended, added or cancelled. Claims 1 and 3 are the independent claims. Claim 10 has been withdrawn from consideration.

Applicants note with appreciation the Examiner's acknowledgement that certified copies of all priority documents have been received by the U.S.P.T.O. Action, summary at 12.

Interview Summary

Initially, Applicant wishes to thank the Examiner for her time during the December 11, 2009 telephone Interview with Applicant's representative Erin Hoffman (Reg. No. 57,752). During the Interview, it was agreed that the presently filed arguments and evidence may overcome the obviousness rejection of independent claims 1 and 3.

Rejections under 35 U.S.C. § 103

Flynn, Widy-Tyszkiewicz, Singh, McClung and Sandoval

Claims 1-7 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the combination of Flynn (The herbal management of stress, Australian journal of medical herbalism, 1996: 8 (1): 15-18), Widy-Tyszkiewicz et al (A randomized double blind study of sedative effects of phytotherapeutic containing valerian, hops, balm and motherwort versus placebo, Herba

polonica, (1997) Vol. 43, No. 2, pp. 154-159), Singh et al (Therapeutic potential of Kava in the treatment of anxiety disorders, CNS drugs 2002: 16 (11): 731-743), McClung (US 6579543) and Sandoval (Cat's claw (Uncaria tomentosa) protects against oxidative stress and indomethacin-induced intestinal inflammation, Gastroenterology, 1997; 112 (4 suppl.): A1081). Applicants respectfully traverse this rejection for the reasons detailed below.

In the Office Action, the Examiner states that it would have been prima facie obvious for one of ordinary skill in the art at the time the invention was made to combine the inventions of Flynn, Widy-Tyszkiewicz et al, Singh et al, McClung, and Sandoval since all of them teach compositions for anti-stress activity individually in the art; that, since all the compositions yielded beneficial results for anti-stress activity, one of ordinary skill in the art would have been motivated to make the modifications to combine the references together; and that thus, the invention as a whole is prima facie obvious over the references, especially in the absence of evidence to the contrary. Applicants respectfully disagree.

Applicants submit that not all of the individual components of Flynn, Widy-Tyszkiewicz, Singh, McClung and Sandoval are used for treatment of stress, and therefore, it would not have been obvious to combine these references. In particular, Applicants respectfully submit that there is a distinction between stress in the sense of mental stress experienced by people and oxidative stress experienced on a cellular level by macrophages as mentioned by Sandoval.

The attached Article I provides a definition of oxidative stress. Oxidative stress is adverse effects occurring when the generation of reactive oxygen species (ROS) in a system exceeds the system's ability to neutralize and eliminate them, and excess ROS can damage a cell's lipids, protein or DNA. Biochemical reactions occur at a great speed and numbers in any cell. During these processes, reactive oxygen species (ROS), such as oxygen radicals, hydrogen peroxide etc, are formed which can react with other compounds present in a cell, thereby causing damage to them. Cells have also developed systems to prevent the ROS of becoming too prevalent in cells creating a balance between the generation and elimination of ROS. If the generation of ROS exceeds the ability of a cell to eliminate them, a situation of oxidative stress occurs. The ultimate consequence thereof is that such a cell dies, such as via apoptosis. Applicants respectfully submit that this process occurs within a system as oxidative stress is not confined to any organism, and is such a basic mechanism which occurs in all cellular systems.

In section 22.8, Article II (the Buchanan article) provides further evidence that oxidative stress results from conditions promoting the formation of active oxygen species that damage or kill cells and illustrates oxidative stress that occurs in plants. Factors that cause oxidative stress include air pollution, oxidant-forming herbicides, heavy metals, drought, heat and cold stress, wounding, UV light and highly intense light conditions that stimulate photoinhibitiion.

In page 561, left and right column, the Article III (the Farr and Kogama article) illustrates what has been learned to date about cellular responses to oxidative stress, primarily in bacteria, e.g., *Escherichia coli* and *Salmonella typhimurium*. The Article IV (the Seib article) describes a list of reactive oxygen species (ROS) "which include the superoxide anion (O_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO·), and the reactive nitrogen species (RNS), which include nitric oxide (NO·) and peroxynitrite (ONOO)." (page 344). Applicants point out to the Examiner that peroxynitrite is used by Sandoval to investigate the cytoprotective properties of *Uncaria tomentosa*. Further, Article IV states that "oxidative stress causes damage to DNA, proteins, and cell membranes and often results in cell death" (page 344). Article V (the Martindale and Holbrook reference) states that "oxidative stress is implicated in various diseases, such as atherosclerosis, diabetes, pulmonary fibrosis, neurodegenerative disorders, and arthritis, and is believed to be a major factor in aging".

Therefore, in view of the above argumentation and evidence, Applicants respectfully submit that the cytoprotective activity of *Uncaria tomentosa* in Sandoval against oxidative stress is different from mental forms of stress, and therefore, cannot be "useful for the same purpose" as the anti-stress activity relating to mental stress conferred by other components in the other cited art documents.

The Applicants, therefore, respectfully request that the rejection to Claims 1 and 3 under 35 U.S.C. § 103(a) be withdrawn.

Claims 2-7, dependent on independent claims 1 and 3, are patentable for the reasons stated above with respect to claims 1 and 3 as well as for their own merits.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection to independent claims 1 and 3 and all claims dependent thereon.

CONCLUSION

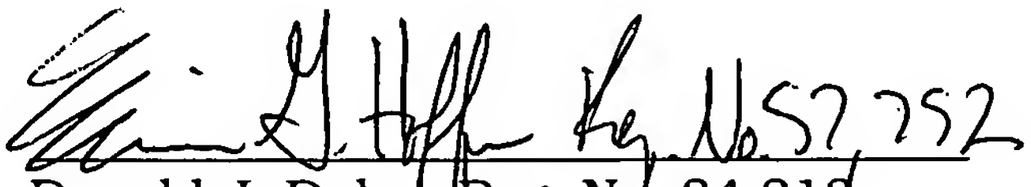
In view of the above remarks and amendments, the Applicants respectfully submit that each of the pending objections and rejections has been addressed and overcome, placing the present application in condition for allowance. A notice to that effect is respectfully requested. If the Examiner believes that personal communication will expedite prosecution of this application, the Examiner is invited to contact the undersigned.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Erin G. Hoffman, Reg. No. 57,752, at the telephone number of the undersigned below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 08-0750 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

HARNESS, DICKEY, & PIERCE, P.L.C.

By  Reg. No. 57,752

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Attachments: Article I (IUPAC Glossary), Article II (Buchanan Article), Article III (Farr and Kogoma Article), Article IV (Seib Article) and Article V (Martindale and Holbrook Article)

Article I

IUPAC Glossary of Terms Used in Toxicology – Terms Starting with O

A B C D E F G H I J K L M N O P Q R S T U V W X Y Z

objective environment
Actual physical, chemical, and social environment as described by objective measurements, such as noise levels in decibels and *concentrations* of air *pollutants*.

occupational environment
Surrounding conditions at a workplace.

occupational exposure
Experience of substances, intensities of radiation etc. or other conditions while at work.

occupational exposure limit (OEL)
Regulatory level of *exposure* to substances, intensities of radiation etc. or other conditions, specified appropriately in relevant government legislation or related codes of practice.

occupational exposure standard (OES)

1. Level of *exposure* to substances, intensities of radiation etc. or other conditions considered to represent specified good practice and a realistic criterion for the control of exposure by appropriate plant design, engineering controls, and, if necessary, the addition and use of personal protective clothing.
2. In GBR, *health*-based exposure limit defined under COSHH Regulations as the *concentration* of any airborne substance, averaged over a reference period, at which, according to current knowledge, there is no evidence that it is likely to be injurious to employees, if they are *exposed* by inhalation, day after day, to that concentration, and set on the advice of the HSE Advisory Committee on *Toxic* Substances.

occupational hygiene
Identification, assessment and control of physicochemical and biological factors in the workplace that may affect the *health* or well-being of those at work and in the surrounding community.

occupational medicine
Specialty devoted to the prevention and management of occupational injury, illness and disability, and the promotion of the health of workers, their families, and their communities.

occupational safety and health
See *occupational hygiene*

octanol-water partition coefficient P_{ow}, K_{ow}

Ratio of the solubility of a chemical in octanol divided by its solubility in water.
Note: Measure of lipophilicity, used in the assessment of both the uptake and physiological distribution of organic chemicals and prediction of their environmental fate.

ocular
Pertaining to the eye.

odds
Ratio of the probability of occurrence of an event to that of non-occurrence, or the ratio of the probability that something is so, to the probability that it is not so.

odds ratio (OR), ϴ
cross-product ratio
relative odds
Quotient obtained by dividing one set of odds by another. The term "odds" or "odds ratio" is defined differently according to the situation under discussion. Consider the following notation for the distribution of a binary *exposure* and a disease in a population or a *sample*.

	Exposed	Nonexposed
Disease	*a*	*b*
No disease	*c*	*d*

The odds ratio (cross-product *ad/bc*.
Note 1: The *exposure*-odds ratio for a set of case control data is the ratio of the odds in favor of exposure among the cases (*a/b*) to the odds in favor of exposure among non-cases (*c/d*), which is equal to *ad/(bc)*. With incident cases, unbiased subject selection, and a "rare" disease (say, under 2% cumulative *incidence* rate over the study period), *ad/bc* is an approximate estimate of the *risk* ratio. With incident cases, unbiased subject selection, and density sampling of controls, *ad/bc* is an estimate of the ratio of the person -time incidence rates (force of morbidity) in the *exposed* and unexposed. No rarity assumption is required for this.
Note 2: The disease-odds (rate-odds) ratio for a cohort or cross section is the ratio of the odds in favor of disease among the exposed population (*a/c*) to the odds in favor of disease among the unexposed (*b/d*), which is equal to *ad/bc* and hence is equal to the exposure-odds ratio for the cohort or cross section.
Note 3: The *prevalence*-odds ratio refers to an odds ratio derived cross sectionally, as, for example, an odds ratio derived from studies of prevalent (rather than incident) cases.
Note 4: The *risk*-odds ratio is the ratio of the odds in favor of getting disease, if exposed, to the odds in favor of getting disease if not *exposed*. The odds ratio derived from a cohort study is an estimate of this.

odor threshold
odour threshold
odor detection threshold
In principle, the lowest *concentration* of an odorant in the air that can be detected by a human being.
Note: In practice, a panel of "sniffers" is often used, and the threshold taken as the concentration at which 50% of the panel can detect the odorant (although some workers have also used 100% thresholds). The odor concentration at the detection threshold may be defined as one odor unit.

oedema
See *edema*

olf
unit used to measure scent emission of people and objects; one olf is defined as the scent emission of an "average person", a sitting adult that takes an average of 0.7 baths per day and whose skin has a total area of 1:8 m^2; the scent emission of an object or person is measured by specially trained personnel comparing it to normed scents.
Note: The olf should not be confused with the of unit of scent immission (as opposed to emission), the *decipol* which also takes into account the ventilation system's air volume flow.

olfactometer
Apparatus for testing the power of the sense of smell.

oligozoospermia
Sperm concentration less than a reference value.
[8]

oliguria
Excretion of a diminished amount of urine in relation to fluid intake.

-omics, -omes
Neologism referring to the fields of study in biology ending in the suffix -omics, such as genomics or proteomics: the related neologism -omes are the objects of study of the field such as the genome or proteome, respectively.

oncogene
Gene that can cause neoplastic (see *neoplasia*) transformation of a cell; oncogenes are slightly changed equivalents of normal *genes* known as proto-oncogenes.

oncogenesis
Production or causation of *tumors*.

oncogenic
Capable of producing tumors in animals, either benign (non-cancerous) or malignant (cancerous).
[9]

one-compartment model
Kinetic model, where the whole body is thought of as a single *compartment* in which the substance distributes rapidly, achieving an *equilibrium* between blood and tissue immediately.
[2]

one-hit model
Dose-response model of the form

$P = 1 - e^{-bd}$

where P is the probability of cancer death from a continuous *dose* rate, d, and b is a constant.

onycholysis
Loosening or detachment of the nail from the nail bed following some destructive process.

oogenesis
Process of formation of the ovum (plural ova), the female *gamete*.

operon
Complete unit of *gene* expression and regulation, including structural *genes*, regulator *gene*(s) and control elements in *DNA* recognized by regulator *gene* product(s).

ophthalmic
Pertaining to the eye.

organ dose
Amount of a substance or physical agent (radiation) absorbed by an organ.

organelle
Microstructure or separated compartment within a cell that has a specialized function, for example ribosome, peroxisome, lysosome, Golgi apparatus, mitochondrion, nucleolus, nucleus.

organic carbon partition coefficient, K_{oc}

Measure of the tendency for organic substances to be adsorbed by soil or sediment, expressed as:

$$K_{oc} = \frac{\text{(mass adsorbed substance) / (mass organic carbon)}}{\text{(mass concentration of absorbed substance)}}$$

The K_{oc} is substance-specific and is largely independent of soil properties.

organoleptic
Involving an organ, especially a sense organ as of taste, smell or sight.

osteo-
Prefix meaning pertaining to bone.

osteodystrophy
Abnormal development of bone.

osteogenesis
Formation or development of bone.

osteomalacia
Condition marked by softening of the bones (due to impaired mineralization, with excess accumulation of osteoid), with pain, tenderness, muscular weakness, anorexia and loss of weight, resulting from deficiency of vitamin D and calcium.

osteoporosis
Significant decrease in bone mass with increased porosity and increased tendency to fracture.

ovicide
Substance intended to kill eggs.

oxidative stress
Adverse effects occurring when the generation of reactive oxygen species (ROS) in a system exceeds the system's ability to neutralize and eliminate them; excess ROS can damage a cell's lipids, protein or DNA.

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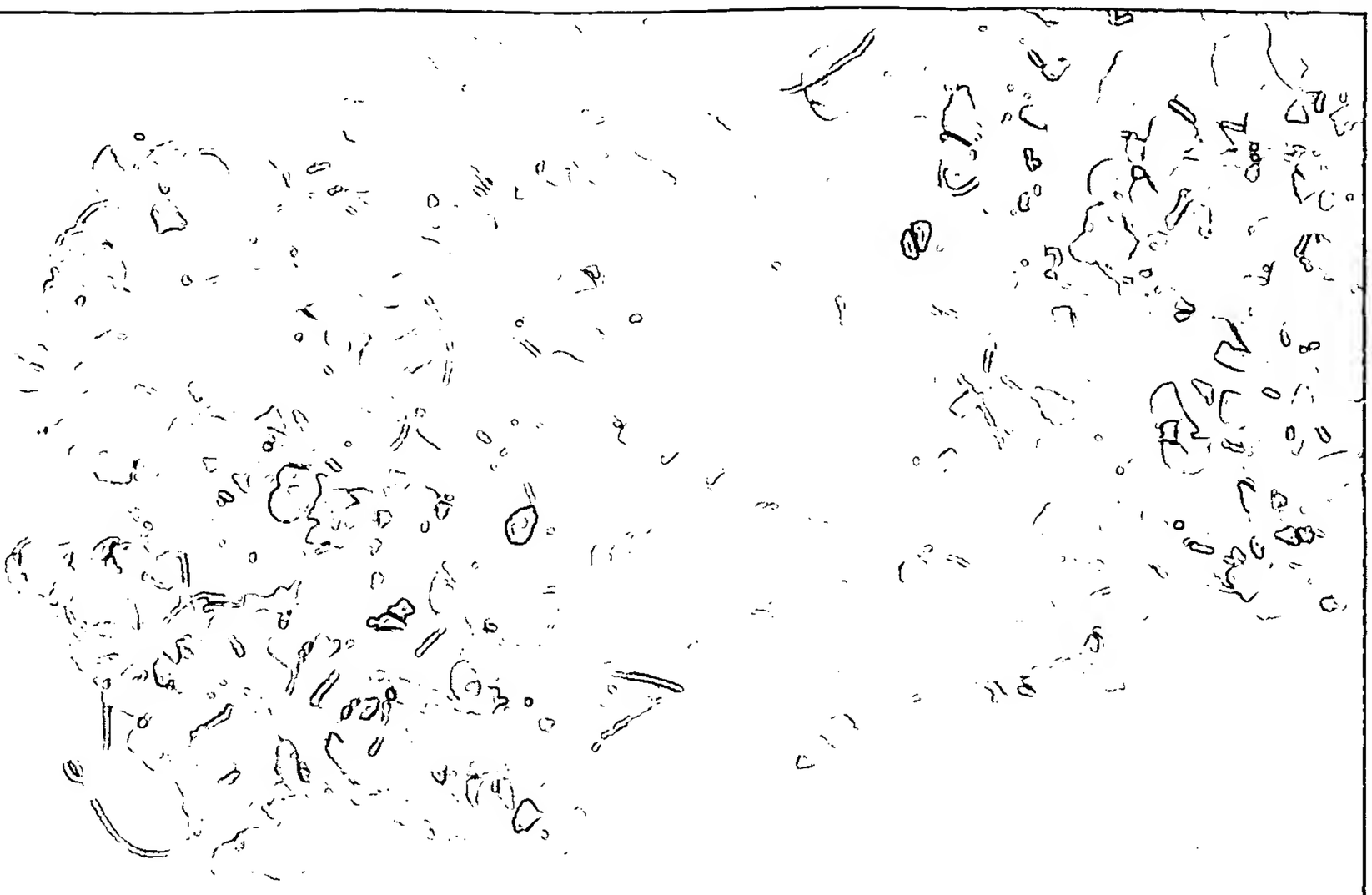
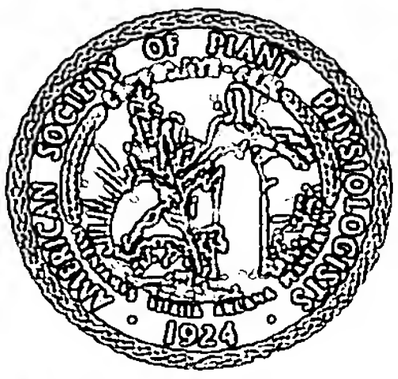
Article II



BIOCHEMISTRY & MOLECULAR BIOLOGY OF PLANTS

BUCHANAN • GRUISSEM • JONES



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curvature caused by cell expansion of the adaxial cells of the petiole, is a common response to waterlogging associated with flood-sensitive plants, such as tomato. Epinasty reduces the foliar absorption of light, thereby slowing transpirational water loss in plants for which water absorption by roots is limited by anoxia. In tomato, severe flooding stimulates transcription of the ACC synthase gene, which results in increased ACC synthesis in the root. In the absence of oxygen, this metabolite cannot be converted to ethylene by ACC oxidase. Some of the ACC is therefore transported to leaves, where ACC oxidase concentrations subsequently increase and ethylene is produced (Fig. 22.34B). This ethylene synthesis results in leaf epinasty. Leaves of tomato plants transformed with an antisense gene complementary to ACC oxidase mRNA demonstrated less foliar ACC oxidase activity, less ethylene production, and less epinasty in response to flooding. These data raise interesting questions regarding the signals transmitted between root and leaf in response to flooding.

22.7.6 How do plants sense oxygen deprivation?

Plant responses to flooding include transient alterations in gene expression and metabolism, as well as long-term developmental responses. How are these responses triggered by low concentrations of available oxygen? The lack of oxygen rapidly results in less ATP and increased NADH as well as decreased cytosolic pH. Any or all of these factors could participate in signal transduction processes. Plant hormones such as ethylene and ABA also may be involved in transducing the low-oxygen signal. In addition, plants possess hemoglobin-like proteins (e.g., leghemoglobin; see Chapter 16), but whether these are involved in sensing oxygen deprivation remains unclear. Constitutive expression of an antisense barley hemoglobin gene in cultured maize cells resulted in decreased hemoglobin content and decreased ability to maintain ATP concentrations, suggesting that hemoglobin may play some role in acclimation to low-oxygen conditions.

Evidence is increasing that Ca^{2+} may be an important second messenger in transducing the low-oxygen signal, altering gene expression, and promoting aerenchyma formation. Anoxia stimulates a rapid increase in cytosolic Ca^{2+} in maize protoplasts. This flux of Ca^{2+}, coming at least in part from mitochondria, appears to be necessary for the increase in *Adh1* transcripts. Use of aequorin, a protein from jellyfish, for fluorescence reporting of cytosolic Ca^{2+} concentrations has provided evidence that a biphasic flux of Ca^{2+} in the response to anoxia in *Arabidopsis* shoots and cotyledons but not in roots. Ca^{2+} has been implicated as a second messenger in the response to heat and cold stress and many other stimuli in plants, and its role in signal transduction is currently a focus of research (see Chapter 18).

22.8 Oxidative stress

Oxidative stress results from conditions promoting the formation of active oxygen species that damage or kill cells. Environmental factors that cause oxidative stress (Fig. 22.35) include air pollution (increased amounts of ozone or sulfur dioxide), oxidant-forming herbicides such as paraquat dichloride (methyl viologen, 1,1′-dimethyl-4,4′-bipyridinium), heavy metals, drought, heat and cold stress, wounding, UV light, and highly intense light conditions that stimulate **photoinhibition** (see Chapters 9 and 12). Oxidative stress also occurs in response to pathogen infection (see Chapter 21) and during senescence (see Chapter 20).

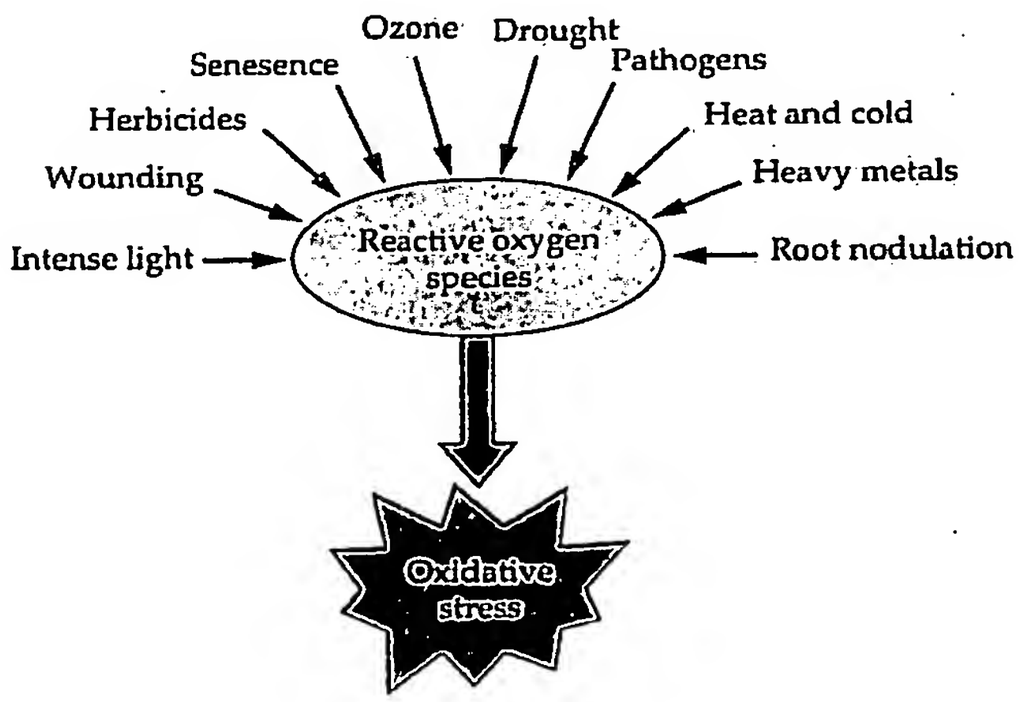


Figure 22.35
Environmental factors that increase the concentrations of reactive oxygen species in plant cells.

Reactive oxygen species (ROS) (Fig. 22.36) are formed during certain redox reactions and during incomplete reduction of oxygen or oxidation of water by the mitochondrial or chloroplast electron transfer chains. Formation of singlet oxygen ($^1O_2$) subsequently stimulates production of other ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl ($HO^{\bullet}$) and perhydroxyl ($O_2H^{\bullet}$) radicals. Superoxide anions also are produced in the chloroplast when electrons are transferred directly from Photosystem I (PSI) to oxygen. These reactive molecules, especially $HO^{\bullet}$, are highly destructive to lipids, nucleic acids, and proteins. Nevertheless, reactive oxygen species such as $O_2^{\bullet-}$ and H_2O_2 are required for lignification (see Chapters 2, 20, and 24) and function as signals in the defense response to pathogen infection (see Chapters 20 and 21). Plants scavenge and dispose of these reactive molecules by use of antioxidant defense systems present in several subcellular compartments. When these defenses fail to halt the self-propagating autooxidation reactions associated with ROS, cell death ultimately results.

The antioxidant defense systems include nonenzymatic and enzymatic antioxidants (Fig. 22.37). These compounds and enzymes are not distributed uniformly, so defense systems vary among specific subcellular compartments (Tables 22.6 and 22.7). The

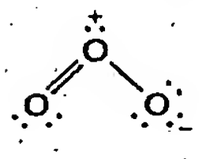
Compound	Shorthand notation(s)	Structural representation(s)	Sources
Molecular oxygen (triplet ground state)	O_2 ; $^3\Sigma$	$:O=O:$ $1s^2 2s^2(\sigma_s)^2(\sigma_s^*)^2(\sigma_x)^2(\pi_y)^2(\pi_z)^2(\pi_y^*)^1(\pi_z^*)^1$	Most common form of dioxygen gas
Singlet oxygen (first excited singlet state)	$^1O_2$; $^1\Delta$	$:O=O:$ $1s^2 2s^2(\sigma_s)^2(\sigma_s^*)^2(\sigma_x)^2(\pi_y)^2(\pi_z)^2(\pi_y^*)^2$	UV irradiation, photoinhibition, photosystem II e⁻ transfer reactions (chloroplasts)
Superoxide anion	$O_2^{\bullet-}$	$[:O=O:]^-$	Mitochondrial e⁻ transfer reactions, Mehler reaction in chloroplasts (reduction of O_2 by iron–sulfur center F_X of Photosystem I), glyoxysomal photorespiration, peroxisome activity, plasma membrane, oxidation of paraquat, nitrogen fixation, defense against pathogens, reaction of O_3 and OH^- in apoplastic space
Hydrogen peroxide	H_2O_2	$H—O—O—H$	Photorespiration, β-oxidation, proton-induced decomposition of $O_2^{\bullet-}$, defense against pathogens
Hydroxyl radical	$OH^{\bullet}$	$:O—H$	Decomposition of O_3 in presence of protons in apoplastic space, defense against pathogens
Perhydroxyl radical	$O_2H^{\bullet}$	$:O=O—H$	Reaction of O_3 and OH^- in apoplastic space
Ozone	O_3		Electrical discharge or UV radiation in stratosphere, reactions involving combustion products of fossil fuels and UV radiation in troposphere

Figure 22.36
Molecular structure of reactive oxygen species active in plants: singlet oxygen, hydrogen peroxide, superoxide anion, hydoxyl radical, and perhydroxyl radical.

major antioxidant species in plants are ascorbate (vitamin C), reduced glutathione (GSH), α-tocopherol (vitamin E), and carotenoids; polyamines and flavonoids also may provide some protection from free radical injury. The ascorbate–glutathione cycle is the major antioxidant pathway in plastids, where ROS are generated during normal biochemical processes that include photosynthetic transfer of electrons. The photosynthetic apparatus receives additional protection from oxidative damage by the exothermic production of the xanthophyll zeaxanthin (see Chapter 12). ROS are produced in root nodules of nitrogen-fixing plants and are scavenged by enzymatic antioxidants. Regulation of the concentrations of antioxidants and antioxidant enzymes constitutes an important mechanism for avoiding oxidative stress.

22.8.1 Tropospheric ozone is linked to oxidative stress in plants.

One of the best characterized causes of oxidative stress is exposure to high concentrations of ozone. Anthropogenic hydrocarbons and oxides of nitrogen (NO, NO_2) and sulfur (SO_x) react with solar UV radiation to generate ozone (O_3). Stratospheric ozone is beneficial because it shields the earth from UV irradiation, but tropospheric ozone is harmful to

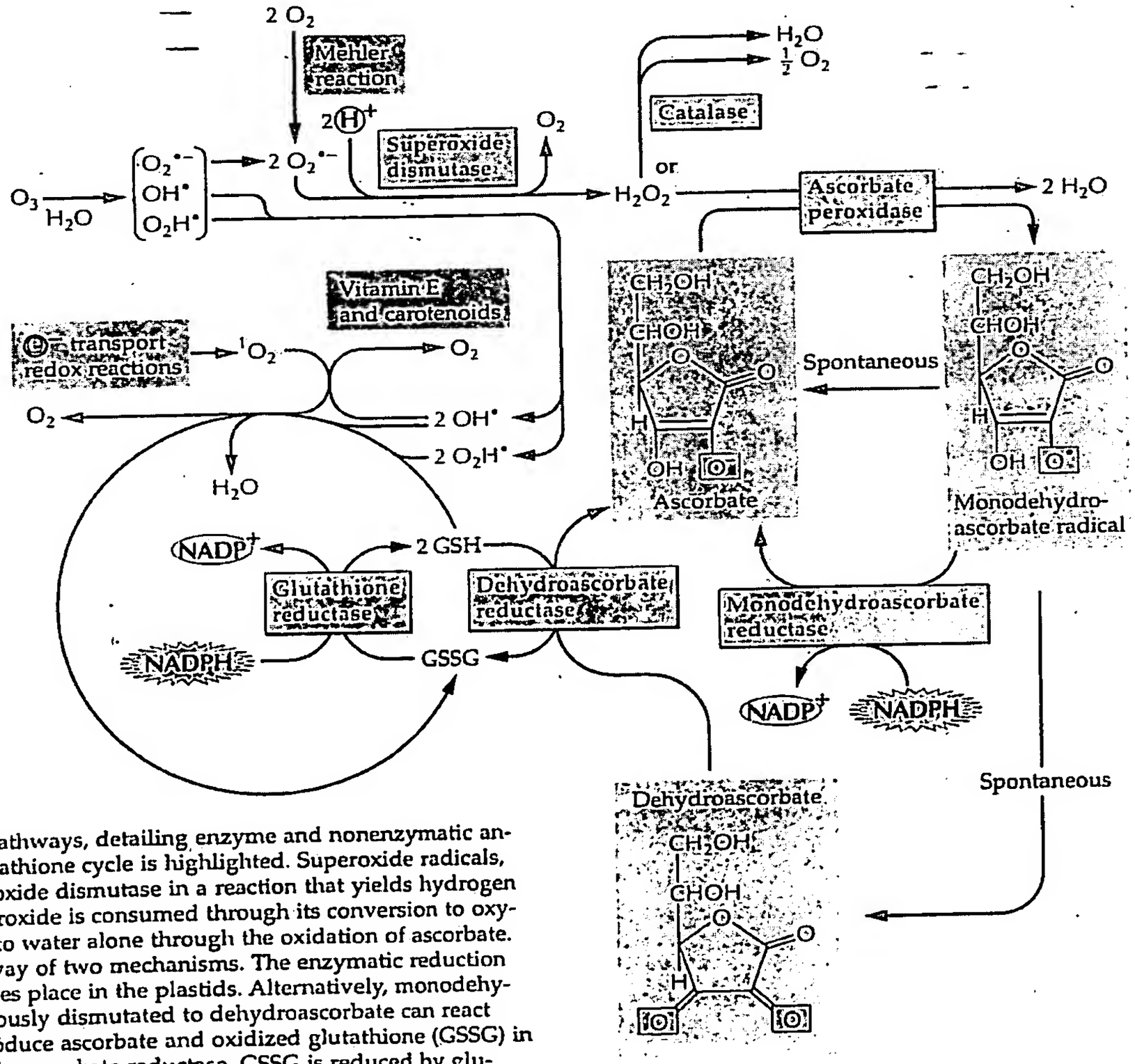


Figure 22.37
Antioxidant defense system pathways, detailing enzyme and nonenzymatic antioxidants. The ascorbate–glutathione cycle is highlighted. Superoxide radicals, $O_2^{\bullet -}$, are eliminated by superoxide dismutase in a reaction that yields hydrogen peroxide, H_2O_2. Hydrogen peroxide is consumed through its conversion to oxygen and water by catalase or to water alone through the oxidation of ascorbate. Ascorbate is regenerated by way of two mechanisms. The enzymatic reduction of monodehydroascorbate takes place in the plastids. Alternatively, monodehydroascorbate that is spontaneously dismutated to dehydroascorbate can react with glutathione (GSH) to produce ascorbate and oxidized glutathione (GSSG) in a reaction catalyzed by dehydroascorbate reductase. GSSG is reduced by glutathione reductase, requiring the consumption of NADPH. Singlet oxygen and hydroxyl ions are eliminated in the glutathione pathway. Damage by singlet oxygen and hydroxyl ions is also diminished by the nonenzymatic antioxidants, vitamin E and carotenoids.

Article III

MICROBIOLOGICAL REVIEWS, Dec. 1991, p. 561–585
0146-0749/91/040561-25$02.00/0

Vol. 55, No. 4

Oxidative Stress Responses in *Escherichia coli* and *Salmonella typhimurium*

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INTRODUCTION AND SCOPE OF REVIEW

Oxidative stress can be functionally defined as an excess of prooxidants in the cell. Active oxygen molecules have been shown to cause damage to DNA, RNA, protein, and lipids. Active oxygen species are produced as an inescapable by-product of normal aerobic metabolism, and their production is further enhanced by exposure to certain environments or by dietary or disease conditions. Oxygen toxicity results when the degree of oxidative stress exceeds the capacity of the cell defense systems. Oxidative stress is

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strongly implicated in a number of diseases such as rheumatoid arthritis, inflammatory bowel disorders, and atherosclerosis (102, 104). It is also emerging as one of the most important causative agents of mutagenesis, tumorigenesis, and aging (1, 3–5, 40, 75). Virtually all aerobic organisms have evolved complex defense and repair mechanisms to mitigate the damaging effects of active oxygen (160, 161).

Because of the powerful genetic techniques available for manipulations in bacteria, tremendous progress has been made toward understanding the genetic and physiological responses to oxidative stress in bacteria. Many aerobic bacteria tested to date appear to encode multigene responses to oxidative stress (for example, see reference 25). In this article, we will summarize what has been learned to date about cellular responses to oxidative stress, primarily in *Escherichia coli* and *Salmonella typhimurium*. We will briefly describe the reactivities of active species of oxygen, detail the genetics and molecular biology of oxidative stress responses, and discuss the physiological role of the responses in oxidative stress. More recently, responses analogous to those first characterized in bacteria have been found in mammalian cells (e.g., 55a, 131, 185a). We will conclude by briefly reviewing the similarities and differences between oxidative stress responses in bacteria and eukaryotic organisms.

This review is intended to be comprehensive and self-contained so that most readers will obtain a current picture of the field without frequent consultation with references. Readers familiar with the background information might skip the beginning section.

Portions of the material covered in this article have been reviewed elsewhere in greater detail. The reader is referred to the following reviews for further coverage of specific topics: the chemistry and biochemistry of oxygen radicals (34, 36, 95, 112, 205); the sources of active oxygen species (3, 74, 188); oxygen radicals and tissue damage, disease, aging, and cancer (75, 98, 104, 198, 207); oxygen radicals and DNA damage and repair (7, 56, 66, 119, 147, 197, 219, 233); lipid peroxidation (61, 79, 80, 103, 129); bacterial responses to other forms of stress (67, 87, 96, 159, 176, 177); and superoxide dismutases (77, 210, 222).

OXIDATIVE STRESS RESPONSES

Active Oxygen Species

Molecular oxygen (O_2) has an even number of electrons. However, it has two unpaired electrons in its molecular orbitals, one in the π^*Y antibonding orbital and one in the π^*Z antibonding orbital; the σ^*Z orbital is empty. These electrons have the same spin quantum number (parallel spins). Molecular oxygen cannot easily oxidize another molecule by accepting a pair of electrons since typically a pair of electrons in an atomic or molecular orbital would have antiparallel spins. As a consequence of its being spin restricted, molecular oxygen is unreactive with most compounds except radicals. Thus oxidation by molecular oxygen is limited to acceptance of electrons. For thermodynamic reasons, molecular oxygen is a poor acceptor of one electron [$E_0(O_2/O_2^-) = -0.33$ V] (see reference 36 and references therein). The spin restriction of molecular oxygen can be overcome by interaction with another paramagnetic center. Transition metals such as Fe or Cu are good catalysts for the one-electron reduction of O_2 (sources of active oxygen species are discussed below).

Its reactivity increases upon acceptance of one, two, or three electrons to form, respectively, a superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$), or when it undergoes a spin flip to become singlet oxygen ($^1\Sigma_g$ O_2) (76, 112). The overall four-electron reduction of molecular oxygen to water is shown below.

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-,\,2H^+} H_2O_2 \xrightarrow{e^-,\,H^+} OH^\cdot \xrightarrow{e^-,\,H^+} H_2O$$

O_2^- (pK_a 4.8) $\rightleftharpoons$ $HOO^\cdot$

Under more acidic conditions, O_2^- can be protonated to form $HOO^\cdot$ (hydroperoxyl radical) (19).

Reactivities. O_2^- will oxidize thiols, ascorbate, tocopherol, and catecholamines (78, 112, 156). Proteins containing $(Fe\text{-}S)_4$ clusters are highly sensitive to attack by O_2^- (80). Probably the most important reactions of O_2^- are its spontaneous dismutation to $H_2O_2 + O_2$ and its ability to reduce transition metals and metal complexes. Spontaneous dismutation of O_2^- in aqueous neutral pH environments produces H_2O_2 (78). As mentioned above, when O_2^- is protonated, the hydroperoxyl radical ($HOO^\cdot$) is produced ($O_2^- + H^+ \rightarrow HOO^\cdot$). The $HOO^\cdot$ radical is much more reactive than O_2^- because the negative charge has been neutralized. The pK_a of this species is 4.8. Thus, at physiological pH, the ratio of O_2^- to its protonated form would be about 100:1. When the pH of the cell (or mitochondria) decreases, such as when oxidative membrane damage has occurred (as will be discussed in a later section), the ratio of $HOO^\cdot$ to O_2^- increases.

O_2^- also can act as a reducing agent for transition metals. In vivo the relevant transition metals are Fe^{3+} and Cu^{2+}, although other transition metals such as titanium can be reduced (34). The general reaction is as follows:

$$O_2^- + M^{(n+1)+} \rightarrow O_2 + M^{n+}$$

The reduction of cupric and ferric ions by O_2^- can occur even when the metals exist in complexed form. For example, cytochrome *c* (Fe^{3+}) can be reduced by cytochrome *c* (Fe^{2+}) by O_2^- (8, 160).

The reactions of H_2O_2 with organic molecules remain unclear, partly because it reacts quickly with contaminating metals to form more reactive species which obscure its own role in oxidation reactions. It can act as a weak oxidizing agent and will attack thiol groups of proteins or reduced glutathione. It can also react directly with some keto acids (104, 237). Most significantly, H_2O_2 will react with reduced iron or copper ions to generate hydroxyl radicals ($OH^\cdot$) in the Fenton reaction (see reference 36 and references therein). Since O_2^- will reduce both Fe^{3+} and Cu^{2+} and since its dismutation produces H_2O_2, it is likely that when the intracellular concentration of O_2^- increases, the concentrations of H_2O_2 and $OH^\cdot$ will also rise.

Reactions of hydroxyl radicals are numerous because $OH^\cdot$ is so reactive that within the cell it will react with most biomolecules at diffusion-limited rates (206). The reactivity of $OH^\cdot$ is due to its very high standard electrode potential, +2.3 V (the standard potential of O_2 is ca. +0.8 V). It will oxidize almost anything but ozone. Because of the reactivity, the average diffusion distance of an $OH^\cdot$ radical is only a few nanometers (206), and thus its effects on any given biomolecule will depend largely upon the location of its formation.

Another form of highly reactive oxygen is ozone (O_3). O_3 is a ubiquitous air pollutant that is formed during photoreactions involving NO_2 and hydrocarbons. Ozone decom-

poses in pure water to form $OH^{\cdot}$ and $HOO^{\cdot}$ radicals with a bimolecular rate constant of 50 to 70 M^{-1} s^{-1} (209). Besides its decomposition to $OH^{\cdot}$ and $HOO^{\cdot}$, ozone reacts rapidly with dienes, amines, and thiols. Mechanisms of ozone reactions with biomolecules include formation of ozonides (cyclic peroxides), or H_2O_2 and aldehydes, or both, through reactions with dienes such as polyunsaturated fatty acids. Ozone may also directly oxidize sulfhydryl (or thiol) groups of amino acids in low-molecular-weight compounds or proteins (166).

Almost all reactions involving singlet oxygen with biomolecules are additions of the $^1O_2$ ($^1\Sigma_g$ O_2) to conjugated bonds. Typically, peroxides are the initial products. $^1O_2$ can add to olefins to produce allylic hydroperoxides and a shift in the double bond, or it can add to diene systems to produce endoperoxides (76). Reactions of $^1O_2$ with unsaturated fatty acids or histidine are good examples of these types of reactions. $^1O_2$ can also undergo (2 + 2) cycloadditions to dienes to produce dioxetanes, which decompose to yield carbonyl compounds (137). The second-order rate constants for the reactions of singlet oxygen with histidine and tryptophan are 1×10^8 and 3×10^7 M^{-1} s^{-1}, respectively (205). Finally, $^1O_2$ will react with α-tocopherol to produce a stable tocopherol radical called chromoxal. Many of the sensitizers which produce $^1O_2$ are embedded within the membrane, and it is possible that $^1O_2$ is capable of initiating lipid peroxidation (129).

In addition to the above active oxygen species, there is growing evidence that iron-oxygen complexes may play a key role in several types of oxidative mechanisms. A superoxide anion will form a complex with Fe^{3+} to yield a "perferryl" radical, $[2Fe^{2+}O_2]^{\cdot}$. This species is not thermodynamically capable of undergoing oxidative reactions with most biomolecules, but will undergo a series of reactions to produce the ferryl radical, $[2Fe^{2+}O]^{\cdot}$ (10, 11, 27, 28). This species is electron rich, has radical characteristics, and is not spin restricted in its reactions. Consequently, it is proposed as one of the major initiating species of lipid peroxidation and possibly DNA damage as well (10, 119). It is worth noting that formation of the perferryl radical will be enhanced under conditions of increased O_2^-.

Sources. Active oxygen species are produced by a variety of compounds and enzymes. The four-electron reduction of O_2 to H_2O is done sequentially and is catalyzed enzymatically by several membrane-associated respiratory-chain enzymes. Recently, Imlay and Fridovich (116) have shown that autoxidation of NADH dehydrogenase, succinate dehydrogenase, and D-lactate dehydrogenase are major sources of O_2^- in *E. coli*. One cytosolic enzyme thought to be capable of generating significant amounts of O_2^- is glutathione reductase, which uses NADH as an electron source (116, 158). Cytochrome P-450s have been shown to be important sources of active oxygen species in mammalian tissues. In certain mammalian tissues, specifically the liver, P-450s can represent up to 4% of the total cell protein (68a). Whereas bacteria contain several P-450s, the role of these cytochromes in production of oxy-radicals is not known.

Nonenzymatic production of O_2^- occurs by autoxidation of several cellular components including ubiquinols, catechols, thiols, and flavins. Electrophilic quinone compounds, both natural cellular constituents (such as ubiquinone) and exogenous sources (such as plumbagin and menadione) (Fig. 1), are easily reduced to semiquinones, which in turn readily reduce O_2 to O_2^-, regenerating the oxidized quinone. The oxidized quinone can undergo this cycle numerous times and is thus referred to as a redox-cycling agent. Paraquat (methyl viologen), a dipyridyl, is also a very effective redox-cycling agent (Fig. 1). As indicated above, reduced transition metals, both free and in complexed form, can donate a single electron to molecular O_2 to generate O_2^-. This reaction is readily reversible and depends upon the concentrations of reduced and oxidized metal and of O_2 and O_2^-.

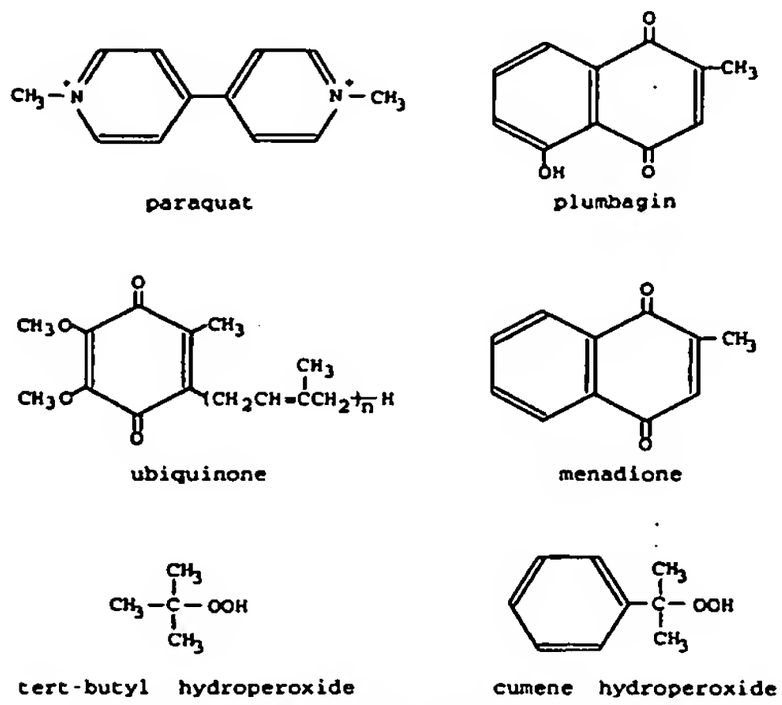

FIG. 1. Commonly used superoxide radical generators and organic peroxides.

Sources of H_2O_2 include spontaneous and superoxide dismutase (SOD)-catalyzed dismutation of O_2^-, as well as several oxidases such as D-amino acid oxidase. There is also abundant evidence that H_2O_2 a photoproduct of near-UV irradiation (67).

Sources of $OH^{\cdot}$ are numerous. Tryptophan residues generate $OH^{\cdot}$ upon absorption of a photon of 365 nm. Radiolysis of water produces $OH^{\cdot}$ as well as a myriad of other active oxygen species (112). Another significant source of $OH^{\cdot}$ is the reaction of H_2O_2 with reduced iron in the Fenton reaction:

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow OH^{\cdot} + H_2O + Fe^{3+}$$

Since O_2^- will reduce transition metals such as iron and since O_2^- is dismutated to form H_2O_2, it is highly likely that when the steady-state concentration of O_2^- rises, the steady-state concentrations of H_2O_2 and $OH^{\cdot}$ will also rise.

Singlet oxygen may be generated in vivo by the action of certain enzymes, decomposition of superoxide, decomposition of peroxidized glutathione ($GSOO^{\cdot}$), and photosensitization reactions with endogenous sensitizers such as riboflavin and bile pigments (76, 205). A combination of the photosensitizer methylene blue and visible-light radiation is also known to generate singlet oxygen in vitro (68).

Oxidative Stress and Cellular Responses

As discussed above, active species of oxygen naturally occur in aerobic cells, arising from a variety of intracellular and extracellular sources. It is not surprising, therefore, that cells maintain a strong defense against the threat. For example, aerobically growing *E. coli* cells are equipped with two superoxide dismutases (SODs), Mn-containing SOD (MnSOD, encoded by *sodA*) and Fe-containing SOD (Fe-SOD, encoded by *sodB*). SODs dismutate O_2^- to H_2O_2. Catalases (HPI catalase encoded by *katG* and HPII encoded

by *katE*) disproportionate H_2O_2 into H_2O and O_2. Alkylhydroperoxide reductase (Ahp; encoded by *ahpC* and *ahpF*) is thought to provide additional defense by reducing various organic hydroperoxides.

Under certain circumstances, the concentration of active oxygen rises to a level that overwhelms the basal level of the scavenging capacity of the cell, giving rise to an oxidative stress condition. Artificially, oxidative stress can be brought about by addition of H_2O_2 or superoxide radical generators such as paraquat and plumbagin (Fig. 1) or by raising the partial pressure of oxygen by bubbling pure oxygen through a culture. Mutational inactivation of genes encoding catalases and superoxide dismutases is also an effective means of imposing oxidative stress onto aerobically growing cells. For example, in *E. coli sodA sodB* double-mutant cells completely lacking SOD activity (37), the concentration of endogenous O_2^- may be elevated 2.5×10^3-fold over that in aerobically growing wild-type cells (116).

Bacteria respond to a variety of stresses and undergo derepression of a set of globally regulated genes (87). At least 13 different multigene systems (stimulons) are known to be induced in the response to a variety of stress stimuli (176). When oxidatively stressed, bacteria respond by invoking one or both of two known stimulons.

Peroxide stress response. In response to an increased flux of H_2O_2 and other organic peroxides such as *tert*-butyl hydroperoxide and cumene hydroperoxide (Fig. 1), the cellular concentrations of at least 30 proteins become elevated over the basal levels. We define this response as the peroxide-mediated stress response, although the actual stimulus for the induction is not known. The peroxide stimulon includes eight proteins in *E. coli* and at least nine proteins in *S. typhimurium* that are positively regulated by the locus *oxyR*. The genes encoding these proteins constitute the OxyR regulon (see below for details). Thus, both *E. coli* and *S. typhimurium* deleted for the *oxyR* gene (*oxyR*Δ) fail to induce these proteins. In OxyR(Con) mutants, the levels of these proteins are constitutively elevated.

Concomitantly with the derepression of the peroxide stimulon, bacteria acquire resistance to peroxide stress. For example, cells pretreated with a low dose of H_2O_2 display enhanced resistance to subsequent challenge doses of H_2O_2 (57). The increased survival derives, at least in part, from an induced DNA repair capacity. Thus, λ phage damaged by exposure to H_2O_2 have a higher plating efficiency on pretreated cells than on naive cells (57). Since λ phage inject naked DNA into the host cells, increased plating efficiency indicates an increased repair capacity for oxidatively damaged DNA. As the OxyR response does not appear to include a DNA repair pathway (70d), this response must be part of the broader peroxide stimulon.

Superoxide stress response. When stressed under elevated levels of the superoxide radical anion, O_2^-, bacteria respond by invoking an entirely different stimulon. Treatment of cells with paraquat, for example, results in induction of more than 30 proteins (91, 234). These superoxide stimulon proteins are for the most part different from those belonging to the peroxide stress stimulon (see below for details). At least six of the superoxide stimulon proteins are known to be regulated by the products of two regulatory genes, *soxR* and *soxS* (92, 226, 241). Mutations inactivating either of the two genes render cells noninducible for the proteins. Thus, SoxR and SoxS proteins positively control the expression of the SoxRS regulon genes.

As in the peroxide stress response, the superoxide stress response enhances survival of the stressed cells. Cells pretreated with a nonlethal dose of plumbagin, for example, display enhanced survival upon an exposure to a challenge dose (71). An increased capacity of pretreated cells for reactivating λ phage damaged by treatment with O_2^--generating agents indicates that the superoxide stress response includes an increased DNA repair capacity (71). This is supported by the finding that the level of endonuclease IV, a DNA repair enzyme belonging to the SoxRS regulon, is dramatically elevated in cells treated with O_2^--generating agents (41).

The two oxidative stress responses are distinct. A large volume of evidence has accumulated which indicates that the peroxide and superoxide stress responses are distinct. For example, cells pretreated with H_2O_2 and cells preinduced with O_2^- generators do not develop cross-resistance; i.e., H_2O_2-treated cells show no enhanced survival when exposed to plumbagin, and vice versa (71). Similarly, H_2O_2-pretreated cells reactivate H_2O_2-damaged phage but not O_2^--damaged phage, whereas O_2^--pretreated cells reactivate O_2^--damaged phage but not H_2O_2-damaged phage. Proteins induced with H_2O_2 are, for the most part, different from those induced under O_2^--generating conditions (234). Furthermore, the OxyR and SoxRS regulons are controlled by two distinct control elements. Most of the OxyR regulon genes are not inducible with O_2^- generators and vice versa. Detailed analyses of the two responses and their regulation are described below.

OXIDATIVE STRESS-INDUCIBLE PROTEINS

Several approaches have been taken to examine proteins and genes induced under oxidative stress conditions. Two-dimensional gel electrophoresis analysis of cell extract from ^{35}S-labeled cells is an effective means of probing induction of proteins (179). Since stress proteins are induced at different times after the onset of stress (44, 228), the timing of protein labeling is a crucial factor in determining the kinds of proteins induced. Also important is the means by which stress is brought about. For example, chronic O_2^- stress brought about by the lack of SOD in aerobically growing SOD^- cells (i.e., *sodA sodB* double mutants) induces proteins that are not seen in wild-type cells stressed by addition of paraquat (234). Treatment with different redox-cycling agents (e.g., paraquat, plumbagin, and menadione), as well as with different concentrations of these agents, results in different patterns of induced proteins (91, 234). These variables would account at least in part for the considerable discrepancy found among reports in the literature.

The second approach is to monitor enzyme activities during the stress (44, 91, 107). Several enzymes have been identified as being induced under various oxidative stress conditions. These are included in Table 1. The third approach is to screen random operon fusions to a reporter gene (e.g., *lacZ*) for inducibility by oxidative stress (132). Since the fusion event often leads to inactivation of the gene, inspection of a two-dimensional electrophoresis gel for disappearance of a protein spot allows one to identify a putative protein which the gene encodes (234). The *soi::lacZ* fusions have been cloned (70d) and partially characterized (70a).

A number of proteins that are induced in *S. typhimurium* and *E. coli* under various oxidative stress conditions have been detected. Table 1 lists some of the oxidatively inducible proteins for which cellular activities or encoding genes, or both, are known. The examination of the patterns of induced proteins permits the following generalization. (i) The proteins that are induced by O_2^- stress are, for the most part,

TABLE 1. Stress proteins and genes inducible by oxidative stress

Protein	Species	Polypeptide	Gene	Inducible[a] by stress (reference):				
				H_2O_2	Paraquat	Menadione	Heat	Nalidixic acid
HPI catalase	*S. typhimurium*	D69/D71	*katG*	+ (44, 171)	?	?	− (171)	− (171)
	E. coli	D78/D78.1	*katG*	+ (91, 228, 234)	− (234)	+ (91)	− (228)	+ (228)
Ahp	*S. typhimurium*	F52a	*ahpF*	+ (44, 171, 212)	?	?	+ (44, 171, 212)	?
		C22	*ahpC*	+ (44, 171, 212)	?	?	− (44, 171, 212)	?
	E. coli	F50.6	*ahpF*	+ (91, 234)	+ (91, 234)	+ (91)	− (228)	− (228)
		B20.9	*ahpC*	+ (91, 234)	+ (91, 234)	+ (91)	− (228)	− (228)
Glutathione reductase	*S. typhimurium*	?[a]	?	+ (44)	?	?	?	?
	E. coli	F45.6	*gor*	?	?	?	?	?
MnSOD	*S. typhimurium*	?	?	+[b] (44)	?	?	?	?
	E. coli	I21.3	*sodA*	− (221), + (228)	+ (91, 108, 221)	+ (91, 108, 221)	?	+ (109)
Endo IV	*E. coli*	F30	*nfo*	?	+ (41, 91, 109, 226, 234)	+ (41, 91, 109, 226)	?	?
Glucose-6-P dehydrogenase	*E. coli*	F48.8	*zwf*	?	+ (91, 128, 226)	+ (91, 128, 226)	?	?
NADPH-dehydrogenase	*E. coli*	?	*ndh*	?	+ (70c)	?	?	?
Soi-28	*E. coli*	F120	*soi-28*	− (132, 234)	+ (132, 226, 234)	?	?	?
Soi-17/19	*E. coli*	H47	*soi-17/19*	− (132, 234)	+ (132, 226, 234)	?	?	?
GroES	*S. typhimurium*	?	?	− (171)	?	?	+ (177)	?
	E. coli	C15.4	*groES*	+ (228), − (91)	+ (91)	+ (91)	+ (228)	+ (228)
GroEL	*S. typhimurium*	C56	?	− (171)	?	?	+ (177)	?
	E. coli	C56.5	*groEL*	− (228), +[c]	+ (234)	?	+ (228)	+ (228)
DnaK	*S. typhimurium*	C69	?	+ (171)	?	?	+ (177)	?
	E. coli	B66.0	*dnaK*	+ (228)	− (234)	?	+ (228)	+ (228)
RecA	*E. coli*	C39.3	*recA*	+ (91, 228)	− (91)	− (91)	− (228)	+ (233)

[a] Symbols: +, inducible; −, not inducible; ?, not known.
[b] Constitutively elevated in *oxyR1* mutants.
[c] L. Shapiro, cited in reference 171.

different from those induced by peroxide stress. (ii) Each stress condition induces a set of proteins including those that are apparently unique to that stress. (iii) Some proteins that are induced by oxidative stress can also be induced by other types of stress, such as heat shock, starvation, and SOS. These points are elaborated in the sections that follow. (iv) The regulation of certain oxidative stress-inducible genes appears to be different in *S. typhimurium* and in *E. coli*. For example, a moderate degree of induction of MnSOD in *S. typhimurium* with H_2O_2 was reported (44), whereas no significant induction was detected in *E. coli* (221). The *ahpF* gene encoding the large subunit of Ahp (Table 1) is induced by heat shock in an OxyR-dependent fashion in *S. typhimurium*, but the cloned *E. coli ahpF* gene cannot be induced by heat in *E. coli* or when introduced into *S. typhimurium* (212). This suggests that the *S. typhimurium* gene has a *cis*-acting heat shock element that is not present in the *E. coli* gene. Curiously, the induction of the *ahpF* gene by heat shock does not depend on the heat shock sigma factor, σ^{32} (212).

Peroxide Stress Proteins

The two-dimensional gel analysis of *S. typhimurium* proteins labeled during the 60-min period following treatment with a low dose of H_2O_2 indicated that the rate of synthesis of 30 proteins is elevated over that seen in untreated cells (44, 171). Of those 30 proteins, 12 (early proteins) are maximally synthesized during the first 30 min whereas 18 (late proteins) continue to be synthesized at an elevated rate in the second 30 min. Nine of the 12 early proteins are constitutively expressed at an elevated level in *S. typhimurium oxyR1* mutants but are not at all induced with H_2O_2 in *oxyRΔ2* strains in which the *oxyR* gene is deleted (44). The *oxyRΔ2* mutation does not affect the inducibility of the remaining 21 proteins. Thus, only 9 of 30 peroxide stress proteins are regulated by the *oxyR* locus. Two of the OxyR-regulated proteins are heat-shock proteins. Four of the remaining seven proteins have been identified; two are electromorphs of the HPI catalase (228), and two are subunits of Ahp (Table 1). As expected, it was found that the activities of these enzymes are constitutively elevated significantly in *oxyR1* mutants (44). In addition, MnSOD and glutathione reductase activities are moderately elevated in the constitutive mutants. It is not known whether any of the remaining unidentified proteins are responsible for these two enzyme activities. Thus, in *S. typhimurium*, 8 to 11 observed proteins are under *oxyR* control. Of course, additional OxyR-regulated polypeptides may be hidden or unobservable on two-dimensional gels.

A similar two-dimensional protein analysis of *E. coli* cells treated with H_2O_2 revealed that more than 30 proteins are synthesized at an elevated rate after induction, 9 of which are constitutively expressed at an elevated level in *oxyR2* mutants (228). Five of the OxyR-regulated proteins are identified: two proteins of HPI catalase, two proteins of *ahp*, and MnSOD (228) (Table 1). However, a study with *sodA-lacZ* operon fusions failed to detect an elevated level of transcription of the *sodA* gene after treatment with H_2O_2 (221). Consistent with this observation, induction of MnSOD was shown to be not dependent on $OxyR^+$ (29). MnSOD is not likely to be induced by peroxide stress in *E. coli*. Thus, in *E. coli*, eight proteins are under *oxyR* control.

O_2^- Stress Proteins

The first enzyme that was identified as being induced by O_2^--generating conditions is MnSOD (93, 107). The activity of endonuclease IV (Endo IV), normally a minor apurinic/apyrimidinic (AP)-endonuclease, was found to be induced dramatically in *E. coli* after treatment with a sublethal dose of paraquat and moderately after treatment with plumbagin and menadione (41). Glucose-6-phosphate dehydrogenase (glucose-6-P dehydrogenase) (91) and NAD(P)H-dehydrogenase (diaphorase) were also found to be induced with paraquat or menadione (Table 1).

A more systematic survey for proteins inducible by O_2^--mediated oxidative stress was undertaken by use of two-dimensional protein gel analysis (234). An increase in O_2^- flux induces MnSOD, which in turn stimulates production of H_2O_2. The elevated level of H_2O_2 is expected to induce peroxide stress-inducible proteins. In this particular study, *sodA sodB* double mutants completely lacking SOD activity were used to minimize the expected secondary induction. Because of their lack of dismutase activity, use of the SOD^- cells also permitted the use of paraquat or plumbagin at approximately 100- to 1,000-fold lower concentrations than those normally used with SOD^+ cells. This was expected to minimize any effect that might arise from the action of the agents other than generation of O_2^- (234). Proteins elevated under these conditions were compared with the proteins induced in SOD^+ cells stressed with O_2^- generators. On the whole, about 30 proteins were found to be induced under O_2^- stress conditions. Of these ca. 30 Soi proteins (for superoxide inducible), 6 have been identified: 2 proteins associated with HPI catalase, 2 proteins of Ahp, heat shock protein GroEL, and endonuclease IV (Table 1). MnSOD, a basic protein, was not resolved on these gels. Except for the two proteins associated with HPI catalase and the two proteins of Ahp, no other H_2O_2-inducible proteins are elevated under O_2^- stress conditions (234). In a similar study, Greenberg and Demple (91) found that O_2^--generating agents, paraquat and menadione, induce in wild-type strains at least 33 proteins that are not seen with H_2O_2 (91). These include GroES and two other heat shock proteins, MnSOD, endonuclease IV, and glucose-6-P dehydrogenase (Table 1).

Screening random operon fusions for inducibility to paraquat treatment, Kogoma et al. (132) found three *soi*::*lacZ* fusions that are inducible specifically with O_2^--generating agents. One fusion, *soi-28*::*lacZ*, lacked the Soi protein F120, and the other two, *soi-17*::*lacZ* and *soi-19*::*lacZ*, did not synthesize the Soi protein H47 with or without O_2^- stress (234). The *soi-28* and *soi-17*/*soi-19* genes have been tentatively assigned to the genes encoding F120 and H47, respectively (Table 1). The functions of these Soi proteins in oxidative stress have not been determined. Since the *soi*::*lacZ* fusion strains are somewhat more sensitive to paraquat than is soi^+ (132), and since the absence of these Soi proteins in the fusion strains results in an increased expression of other Soi proteins (234), they are possibly protective proteins involved in scavenging active oxygen species.

A recessive mutation in the locus, *mvrA*, that renders cells sensitive specifically to paraquat (methyl viologen) has been isolated (172). The wild-type gene has been cloned, and the nucleotide sequence has been determined. The molecular weight and pI estimated for the amino acid sequence are similar to those of the Soi protein H30 (234). However, inducibility of MvrA protein with paraquat has not been demonstrated.

There are conflicting reports with regard to the inducibility of OxyR-regulated proteins with O_2^- generators. Hassan and Fridovich (108) detected significant induction of catalase activity with menadione and plumbagin but not with paraquat, whereas Kao and Hassan (128) reported induction of catalase with paraquat. Greenberg and Demple (91) observed the induction with paraquat or menadione of all of the proteins that are under OxyR control. The induction of OxyR proteins including HPI and Ahp proteins with paraquat, plumbagin, or menadione is clearly $oxyR^+$ dependent (91, 234). This suggests that the induction is mediated by H_2O_2, a product of the dismutation of O_2^- which is stimulated by induced MnSOD as discussed above. In keeping with this suggestion, Walkup and Kogoma (234) found that depletion of SOD activity as in *sodA sodB* double mutants abolishes the induction of these proteins with paraquat or plumbagin, with the exception of AhpF, which is present at a markedly elevated level in SOD^- cells with or without paraquat treatment. Greenberg and Demple (91) also reported significant levels of induction of HPI catalase proteins in SOD^- cells treated with menadione. It appears, therefore, that a significant amount of H_2O_2 is generated from O_2^- in the complete absence of SOD activity in *sodA sodB* double mutants. However, the possibility cannot be ruled out that the redox-cycling agents commonly used may generate H_2O_2 directly. Alternatively, some of the OxyR regulon genes may possess a *cis*-acting control element that is sensitive to an O_2^--mediated inducing signal (see below).

In summary, more than 30 proteins are induced under O_2^- stress conditions in *E. coli*. These proteins are, for the most part, different from those induced by H_2O_2 stress. For at least six superoxide stress proteins, enzyme activities and/or encoding genes are known (Table 1). It should be noted that there are no comparative studies for *S. typhimurium*. Therefore, we know little about proteins inducible by O_2^- stress in this organism, with the exception of MnSOD (44).

Overlaps with Other Stress Responses

Heat shock response. One of the remarkable observations, repeated in a number of studies, is that oxidative stress induces some of the proteins that are also induced under other stress conditions such as heat shock and DNA damage (44, 91, 171, 228, 234). In particular, heat shock proteins GroES and GroEL have been shown to be induced by both peroxide- and superoxide-mediated oxidative stresses as well as heat shock, starvation, and SOS, at least in *E. coli* (Table 1). Another heat shock protein, DnaK, has been shown to be induced by treatment with H_2O_2, nalidixic acid, UV irradiation, and starvation. Elevated levels of the protein, however, could not be seen under O_2^- conditions (234). An SOS protein, RecA, can be induced by both types of oxidative stress but not by heat shock (Table 1). These observations clearly indicate that several stress responses overlap and that the extent of the overlap varies in different overlapping responses.

Mutants of *E. coli* deleted for the *rpoH* gene, which encodes the major heat shock sigma factor, σ^{32}, are extremely sensitive to thermal stress; they cannot grow at temperatures above 20°C (246). Heat shock genes are expressed at much reduced rates in these mutants. However, overproduction of GroEL and GroES proteins alleviates the effects of the *rpoH* deletion and allows growth at up to 40°C (136). Growth at temperatures above 40°C requires concomitant overexpression of the *dnaK* gene (136). These results strongly suggest that GroE and DnaK are key proteins that

are important in protection against thermal stress. Together with these observations, the fact that these proteins are induced in at least four overlapping responses (Table 1) points to an attractive notion that some of the heat shock proteins may serve as general antistress proteins. In support of this idea, it has been demonstrated that the *rpoH* deletion renders *E. coli* SOD^+ and SOD^- cells extremely sensitive to both peroxide and superoxide stress, although a mutation in the *groES*, *grpE*, or *dnaK* gene does not by itself increase the sensitivity appreciably (132a).

Privalle and Fridovich (186) observed that heat shock (for 1 h at 48°C) followed by a period of recovery at 37°C resulted in a moderate induction of MnSOD. Since the induction was seen only under aerobic conditions, they speculated that heat shock might generate O_2^- as a result of disruption of the electron transport system of the membrane by heat. In support of this proposal, a recent study has shown that certain membrane-binding drugs (anesthetics) which inhibit the membrane-bound respiratory activity also induce MnSOD only under aerobic conditions (245). Whether these cells acquired resistance to heat shock or oxidative stress was not examined.

Cells adapted to peroxide stress exhibit increased resistance to heat shock. Thus, pretreatment of *S. typhimurium* with H_2O_2, which induces 30 proteins including 5 heat shock proteins (e.g., DnaK) (171), leads to markedly increased resistance to thermal stress (44). An OxyR(Con) mutant (*oxyR1*) of *S. typhimurium* was shown to be markedly more resistant to killing by heat than the wild type was (44). Similarly, transcription of several heat shock genes has been found to be constitutively elevated in *E. coli* SOD^- cells chronically stressed by high levels of O_2^-. However, these cells are no more resistant to thermal stress than are SOD^+ cells, partly because they are defective in the induction of heat shock genes upon temperature shift-up (132a).

Carbon starvation response. Glucose-starved stationary-phase cells of *E. coli* develop enhanced resistance to several forms of stress such as heat shock, oxidation, osmotic shock, and starvation, and more than 30 proteins are induced in starved cells, including several oxidative stress and heat shock proteins (126, 159). Apparently, three of the heat shock proteins (DnaK, GroEL, and HtpG) are regulated by σ^{32}, which has been shown to increase upon starvation (125a). However, the oxidative stress proteins seen in starved cells are not induced by oxidative stress. Instead, the induction of these proteins is regulated as part of a multigene system (the KatF regulon) which is turned on upon entry to the stationary phase or to carbon starvation (see below).

SOS response. Genetic stress invokes the SOS response, which includes an inducible DNA damage repair response (233, 239). Brawn and Fridovich (30) reported that paraquat treatment causes induction of a *din* (damage-inducible) gene and imparts resistance to UV irradiation only in the presence of oxygen. They proposed that O_2^- induces the SOS response. However, induction of MnSOD with paraquat has since been shown to be independent of $RecA^+$ (105). Plumbagin at the concentrations that induce a DNA repair response in *E. coli* has failed to activate the *din* gene or to confer resistance to UV irradiation (71). The concentrations of paraquat necessary to confer protection against UV irradiation (0.5 to 10 mM) (30) are much higher than those needed to fully induce SoxRS regulon genes (e.g., 0.1 mM for *sodA* and 0.05 mM for *soi* genes) (105, 132, 221). It is possible, therefore, that paraquat at only high concentrations causes SOS-inducing damage (e.g., single-strand breaks) by elevating both O_2^- and H_2O_2 concentrations, leading to formation of $OH^\cdot$. The $RecA^+$ dependence of the induction of UV resistance with paraquat is yet to be demonstrated.

Treatment with low (91) but not high (101) concentrations of H_2O_2 induces *recA*, an SOS gene. Similarly, H_2O_2 is known to cause the induction of two SOS functions, λ phage induction from lysogens and cell filamentation (118). Cell filamentation during the SOS response is due to the induction of the *sfiA* (*sulA*) gene encoding a cell division inhibitor (82). It is puzzling, therefore, that the H_2O_2-induced filamentation is not dependent on $sfiA^+$ (118) and yet the *sfiA* gene is inducible with H_2O_2 (65, 84). It is likely that H_2O_2 causes both SOS-dependent and SOS-independent inhibition of cell division.

REGULATION OF CELLULAR RESPONSES TO OXIDATIVE STRESS

OxyR Regulon

Positive regulatory protein, OxyR. The expression of a limited number of the proteins inducible with H_2O_2 is regulated by the *oxyR* locus. Thus, *oxyR* controls only a subset of the 30 H_2O_2-inducible proteins. This was demonstrated by the isolation of an *S. typhimurium* mutant, *oxyR1*, in which the expression of 9 of the early 12 proteins is constitutively elevated (44). The *oxyR1* mutation was mapped by linkage to Tn*10* insertions in the nearby *argH* (at 89.5 min on the standard *E. coli* linkage map [12]). By Tn*10*-mediated deletion, *S. typhimurium* and *E. coli* mutants deleted for the *oxyR* locus were generated (44). The deletion of the gene abolished the inducibility of the nine proteins with H_2O_2. Thus, it was inferred that the *oxyR* gene product is a positive factor essential for activation of the OxyR regulon genes (44). The *E. coli oxyR* gene has been cloned, and the nucleotide sequence has been determined (45, 216a). The gene encodes a protein of 34 kDa which shares significant homology with a family of bacterial regulatory proteins known as the LysR family (111). The *oxyR* gene expression is negatively autoregulated, as is the expression of many Lys family regulatory genes (45, 216a).

The regulation by OxyR operates primarily at the transcriptional level. Thus, the *katG* transcript is elevated more than 50-fold in *S. typhimurium oxyR1* mutants (171), which is in good agreement with the 50-fold increase in catalase activity observed in the mutants (44). Induction of β-galactosidase with H_2O_2 in strains carrying a *katG*::*lacZ* operon fusion is completely blocked by introduction of an *oxyR* deletion mutation (218). Similar transcriptional activation of the *ahp* operon by OxyR has been demonstrated by the presence of elevated levels of *ahpC* transcript and AhpC protein in *oxyR* constitutive mutants (218). Footprinting analysis with purified OxyR protein has identified the regions upstream of the *katG* gene, *ahpCF* operon, and *oxyR* gene to which OxyR protein binds. The OxyR-binding sites extend into the -35-σ^{70} binding sites, suggesting that the OxyR protein interacts with RNA polymerase to activate transcription. Intriguingly, inspection of the sequences of these OxyR-binding sites has revealed only a few conserved nucleotides (218).

OxyR is known to regulate the expression of one other gene: the phage Mu *mom* gene which encodes a DNA modification function (26). OxyR (MomR) protein prevents transcription of the *mom* gene by binding to a 5′ region of the *mom* structural gene which contains three GATC sites (the

target sequence for Dam methylase [157]), only when these sites are unmethylated. Thus, the OxyR protein can discriminate between unmethylated and methylated states of the *mom* promoter region (26). Whether the Dam methylation-dependent repression of the *mom* gene in any way relates to oxidative stress is not known. *E. coli dam* mutants are hypersensitive to killing by H_2O_2 (242). Thus there may be other OxyR-regulated genes whose repression or induction depends upon correct methylation. It is interesting that there is significant homology between the OxyR-binding site sequences of the *mom* and *oxyR* genes (26), in contrast to the absence of a consensus among those of the *katG*, *ahpCF*, and *oxyR* genes as mentioned above. This suggests that the modes of interaction with DNA might be different depending on whether the protein functions as a repressor or an activator.

Direct activation of OxyR protein by oxidative stress. In response to the elevated flux of H_2O_2, OxyR protein activates transcription of the OxyR regulon genes. How is an oxidative stress signal transduced to OxyR protein? Upon treatment of cells with H_2O_2, the transcription of *oxyR'-'lacZ* does not change (216b), nor does the rate of OxyR protein synthesis increase (213). Thus, induction of the OxyR regulon does not involve an increase in the amount of OxyR expression. The possibility of direct activation of OxyR protein by oxidation was suggested by the initially surprising observation that OxyR protein purified from unstressed cells is capable of activating transcription of *ahpCF* and *katG* genes in vitro. It was subsequently demonstrated that OxyR prepared in the absence of oxygen is inactive as a transcriptional activator, but it can be readily converted to an active form by exposure to air (213). In fact, addition and removal of dithiothreitol, a reducing agent, permits conversion between the active and inactive forms of the protein. The conversion to the active form by removal of the reductant can be prevented by the addition of catalase. Therefore, it appears that as soon as it is removed from the reducing environment within the cell, OxyR protein is oxidized, perhaps by H_2O_2 that is present under the aerobic conditions. These results led to the conclusion that upon oxidative stress the increased flux of H_2O_2 converts OxyR protein to an oxidized form, which in turn activates transcription of the OxyR regulon genes (213). Thus, in this scenario, OxyR is both the sensor and the transducer of an oxidative stress signal which is H_2O_2 itself.

The mechanism by which the oxidized form of OxyR activates transcription has not been clearly elucidated. OxyR protein contains six cysteine residues (45). Although good candidates for the redox-active center, these cysteine residues are not likely to be involved in formation of inter- or intramolecular disulfide bonds by oxidation because the conversion of five of the six residues to serine does not affect the activation of the protein (213). A change in the cysteine residue at position 199 to serine inactivates the protein, however. Both the reduced and oxidized forms of OxyR bind to the regulatory regions of *ahpCF*, *katG*, and *oxyR*, but the footprints obtained with the two forms are clearly different, suggesting a distinct conformational change upon oxidation and reduction of the protein (213). OxyR mutant protein, which is inactivated by conversion of the essential cysteine residue to serine, even when prepared in the presence of oxygen, yields a footprinting pattern identical to that seen with the reduced form of wild-type protein. It has been suggested that the postulated conformational change alters the interaction of the protein with RNA polymerase, leading to activation of transcription (213). It should be pointed out, however, that the same difference in the footprinting patterns between the reduced and oxidized forms can be seen with the regulatory region of the OxyR regulon gene which is bound and repressed by both the reduced and oxidized forms of OxyR (213). Therefore, the suggested conformational alteration may be a necessary change, but not a sufficient event leading to transcriptional activation. The understanding of the precise mechanism of the transcriptional activation would require detailed structural and genetic studies of the two forms of the OxyR protein.

SoxRS Regulon

Isolation of Sox(Con) mutants. The demonstration that the induction of *soi::lacZ* fusions is completely independent of previously described regulatory loci, *oxyR*, *rpoH*, and *recA*, led to the prediction of the existence of a new control locus termed *soxR* (for superoxide radical response) (132). The *soxR* regulatory locus was defined by subsequent isolation of mutations [SoxR(Con)] that render the expression of several O_2^--inducible genes constitutive in *E. coli*. Two mutations, *soxR1* and *soxR2*, were isolated by searching for extragenic mutations that elevated the expression of an *nfo'-'lac* fusion (226). Additionally, two mutations, *soxR101* and *soxR105*, were found among the mutations that conferred elevated levels of resistance to redox-cycling agents (92). The mutations have been mapped at 92.2 min on the *E. coli* chromosome (92, 226). The genes which are expressed constitutively in these mutants are *nfo*, *zwf*, *sodA*, *soi-17*, *soi-19*, and *soi-28* (Table 1). Four other proteins including a ribosomal protein were also found elevated in *soxR*(Con) mutants by two-dimensional gel analysis (92). The SoxR(Con) mutants are not particularly more resistant to O_2^--generating agents than is the wild type, except for *soxR101* and *soxR105* mutants, which are isolated by virtue of increased resistance to menadione. Thus, the mutants are resistant to this agent and plumbagin, but not to paraquat (92).

The regulation by the *soxR* locus appears to occur primarily, if not exclusively, at the level of transcription because SoxR(Con) mutations affect the expression of *nfo'-'lacZ*, which utilizes the ribosome-binding site of *lacZ* (226). Large deletions in the *soxR* locus lead to noninducibility by paraquat. The inducibility can be restored by introduction of a plasmid carrying this region of the chromosome (92, 226). Thus, the *soxR* locus encodes a *trans*-acting positive factor(s) essential for transcriptional activation of *soxR* regulon genes.

The *soxR* region encodes two proteins. The actual SoxR regulation appears to be much more complex than one positive factor activating transcription of the regulon in the response to stress. By analyzing proteins coded for by the DNA sequences in this region, Tsaneva and Weiss (226) have detected two polypeptides, of 17 and 13 kDa, which they have assigned to SoxR and SoxS proteins, respectively. Both SoxR and SoxS are essential for the inducibility of the SoxR regulon genes (241). Deletions and insertions near the 3′ end of the soxR gene lead to the constitutive phenotype, thus raising the possibility that the activation of the SoxR protein involves cleavage by a protease activity in analogy to the RecA-mediated cleavage of LexA protein in SOS induction (233). The two genes, *soxR* and *soxS*, are transcribed divergently (226). Furthermore, the *soxR* promoter lies within the *soxS* gene (241). The significance of this divergent and overlapping transcription has not been elucidated. The mechanism by which the two proteins activate transcription of the SoxRS regulon genes is not known. One possibility

that the activation involves an increase in the amount of these proteins has been suggested by the observation that the *soxS* gene is inducible by paraquat (241). Since SoxR is normally expressed at barely detectable levels (226), the expression of *soxR* may also be inducible by O_2^- stress.

The individual roles of the SoxR and SoxS proteins in the regulation of SoxRS-regulated genes has not been elucidated. SoxS is related to the AraC family of proteins (2a, 241) that function as positive transcriptional regulators. The SoxR protein was found to share regions of homology to the MerR protein (2a), which, when bound by mercury, positively regulates several genes involved in mercury detoxification (180). SoxR contains four cysteines clustered in a region near the carboxy terminus (241). This suggests the possibility of a bound metal whose redox state might determine the activity of the SoxR protein as an inducer (signal sensor) or transcriptional activator. It has recently been shown that SoxS alone can switch on the SoxRS regulon genes in the absence of SoxR or paraquat (2a).

Pleiotropic effects of *soxR*(Con) mutations. Greenberg et al. (92) have made an interesting observation that SoxR(Con) mutants are multiply resistant to several antibiotics including chloramphenicol, nalidixic acid, ampicillin, tetracycline, and bleomycin. This multiple drug resistance phenotype can be accounted for at least in part by additional effects of *soxR*(Con) mutations on gene expression. First, SoxR(Con) mutants have diminished amounts of protein S6A, accompanied by concomitant increases in protein S6C (92). The gene encoding this activity (*rimK*) has recently been cloned and sequenced (127). Addition of glutamyl residues to the carboxy terminus of the S6 protein, yielding S6C, affects the level of resistance to several antibiotics (189). Second, the levels of the OmpF porin are significantly decreased in SoxR(Con) mutants (92). This change appears to be mediated by the increased expression of the *micF* gene (55a) encoding the antisense RNA, which inhibits translation of *ompF* mRNA (169). A similar multiple drug resistance conferred by the *marA* mutations has been shown to be caused at least in part by the decreased levels of OmpF porin in these mutants (46). It appears, therefore, that *soxR*(Con) mutations enhance the expression of the *micF* gene which decreases the OmpF porin level and in turn lowers the membrane permeability to antibiotics.

Garvey et al. (81) observed a significant decrease in the level of OmpF porin upon SOS induction by treatment with nalidixic acid. Since nalidixic acid treatment is known to induce some oxidative stress proteins (228), Greenberg et al. (92) speculated that nalidixic acid might generate oxygen radicals in the treated cells. This attractive hypothesis is unlikely because the decrease in OmpF porin is observed not only after SOS induction in *recA*$^+$ cells treated with nalidixic acid, but also in *recA730* mutants which constitutively express SOS genes in the absence of nalidixic acid (81).

Other Factors Involved in Regulation of SoxRS Regulon Genes

There is evidence indicating that SoxRS regulon genes are regulated by additional factors. For example, SoxRS regulon genes are not maximally expressed in SoxR(Con) mutants; thus, the expression of several SoxRS regulon genes can be further elevated by treatment of SoxR(Con) mutants with paraquat (92, 226). This partial expression might account for the puzzling observation that *soxR*(Con) mutants are not particularly more resistant to many redox-cycling compounds than is the wild type, as mentioned above. A group of mutations [*soxQ*(Con)] have been isolated which elevate the expression of some SoxRS regulon genes at the transcriptional level like *soxR*(Con) but map at a locus distinct from the latter (88). *soxQ*$^+$ is not required for the induction by paraquat. Thus, SoxQ could be a factor responsible for additional control of some SoxRS genes.

Multilayered Regulation of SodA Expression

In addition to the SoxRS control, the expression of the *sodA* gene is under at least two and possibly three other global controls. Moody and Hassan (170) made a seminal observation that MnSOD can be anaerobically induced by the addition of iron chelators. The observation led them to propose the involvement of an iron-containing protein in the negative control of the SodA expression at the transcriptional level. Niederhoffer et al. (178) noted that the promoter region of the *sodA* sequence (216) contains a stretch of sequence that is homologous to the iron box, a 19-bp consensus sequence that is found in the regulatory region of genes involved in iron uptake (14). The Fur protein which requires Fe^{2+} as cofactor binds to the iron box and represses the expression. The involvement of Fur protein was subsequently demonstrated by modest levels of derepression of the *sodA-lacZ* operon fusion in aerobically and anaerobically growing *fur* mutants and by gel shift of DNA fragments containing the *sodA* promoter by Fur protein (178).

The genetic study by Tardat and Touati (217) confirmed the involvement of Fur and further uncovered another layer of control over SodA expression. They isolated and analyzed mutants that are capable of anaerobic expression of SodA. The analysis revealed that high levels of the anaerobic derepression require two mutations, one each in the *fur* and *arc* regulatory systems, and that single mutations affecting only one of the systems allow only partial derepression. The *arc* regulatory system negatively controls expression of a number of genes involved in aerobiosis (121–123). Thus, SodA expression is coupled to respiration by the Arc regulation and to iron availability by the Fur system. It has been speculated that the derepression of MnSOD by the unavailability of ferric iron is designed to compensate decreased activities of FeSOD in case of insufficient cellular iron concentrations for full FeSOD activity (217). Importantly, the derepressed level of MnSOD in *fur arc* double mutants can be further elevated by paraquat in the presence of oxygen, indicating that the positive regulation by SoxRS is independent of the Fur and Arc systems (217). Recent evidence suggests a role for Fnr in SodA expression as well (109a). Thus, the SodA expression is under at least three independent global regulatory systems. In addition, evidence indicates that overproduction of MnSOD represses transcription of the *sodA* gene; thus, the SodA expression is also autoregulated (221).

KatF (RpoS) Regulon

E. coli possesses two catalases, HPI and HPII, encoded by *katG* and *katE*, respectively (152, 154). Whereas KatG catalase expression is under OxyR control, KatE expression is not inducible with H_2O_2, but is regulated in a growth phase-dependent manner (201). The synthesis of KatE catalase requires the *katF* gene product as a positive regulatory factor (153). The nucleotide sequence of a cloned *katF* gene has suggested that KatF protein is a σ factor (173). KatF synthesis is turned on before or coincidently with KatE expression as cells enter the stationary phase (174). Thus, it

appears that a starvation signal triggers the synthesis of KatF, which activates RNA polymerase to turn on transcription of the *katE* gene. Moreover, the observation that *katF* mutants die off more rapidly than the wild type upon prolonged exposure to starvation conditions led to the suggestion that KatF positively controls other genes involved in the response to starvation stress (174).

Exonuclease III (Exo III) encoded by *xthA*, is an important repair enzyme in H_2O_2-mediated oxidative stress (58, 59). Thus, *xthA* mutants are very sensitive to exposure to near-UV and H_2O_2 (58, 194). Although KatF regulates KatE expression, inactivation of the *katF* gene but not *katE* results in sensitivity to near-UV (195). This observation led Sak et al. (193) to examine a possible role of KatF in XthA expression. They found that *katF* mutant cells contain as little Exo III activity as *xthA* mutant cells. The expression of *xthA* is controlled in a growth phase-dependent manner, as is *katE* expression. Thus, KatF appears also to regulate XthA expression. The speculation that the *xthA* and *katE* genes belong to the same operon (193) has not been critically examined.

Furthermore, *katF* (*appR*) is also known to regulate the expression of the *appA* gene (138, 225), which encodes an acid phosphatase (224), and the *bolA* gene (22, 139), a "morphogene" which controls the cell morphology change from the rod shape of growing cells to the spherical shape upon entry to the stationary phase (2). It is not likely that these gene products play a significant role in the defense against oxidative stress. Instead, the *katE* and *xthA* genes are regulated as part of a multigene system (the KatF or RpoS regulon) which is invoked when cells enter the stationary phase (138, 159). The induction of *katE* and *xthA* would account for the remarkable degree of tolerance against H_2O_2 which develops in glucose-starved stationary-phase cells (126, 159a) since *katF* is one of the carbon starvation-inducible genes (138). Glucose starvation also elicits resistance to heat shock (126). However, *katF* is not inducible by heat (138). Thus, it appears that stationary-phase cells can develop tolerance to several types of stress by a unique mechanism which is not mediated by regular stress response systems such as the OxyR and heat shock regulons.

AppppN, Heat Shock, and Oxidative Stress

Ames and coworkers found that unusual nucleotides, AppppN and ApppN (N = A, C, G, or U), accumulate in cells stressed by heat shock or by exposure to a wide variety of oxidizing agents including menadione and H_2O_2 (141). An enzyme (dinucleotide tetraphosphate hydrolase) which hydrolyzes the nucleotides has been identified, and the gene (*apaH*) encoding the enzyme has been cloned (164). It has been proposed that these nucleotides may be alarmones signaling the onset of the stress (21). At least three lines of evidence resulting from subsequent investigations appear to rule out this possibility. First, the time course of the accumulation of AppppN lags behind induction of heat shock proteins (228). Second, cells carrying an *apaH*$^+$ gene on a high-copy-number plasmid do not accumulate the nucleotides during heat shock or oxidative stress as a result of an elevated level of the hydrolase activity. However, the pattern of the proteins induced under stress conditions is not altered (184). Nor are ApaH overproducers particularly sensitive to H_2O_2 (184). Third, *apaH*(Null) mutants, whose basal level of AppppN is the same as that reached in heat shock-induced wild-type cells, do not show elevated levels of constitutive expression of heat shock proteins (69).

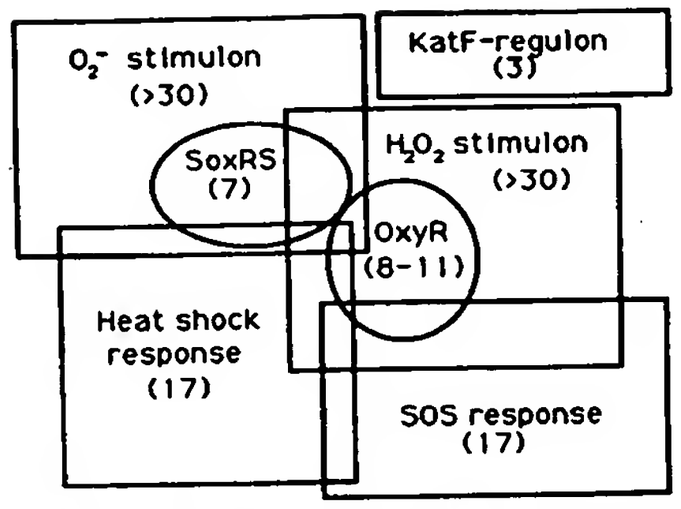

FIG. 2. Cellular responses to oxidative stress and possible overlaps with other stress responses. The number in parentheses indicates the number of proteins and genes known to be involved in the response. Recent studies (159, 159a) indicate that the KatF regulon may contain more than 30 proteins. See the text for details.

Although AppppN cannot be a signal for the heat shock or oxidative stress response, the following evidence (69, 126a) suggests that AppppN levels may modulate the heat shock response, perhaps by affecting DnaK functions. (i) *apaH* (Null) mutants are more sensitive to killing at 55°C than is the parental strain with or without preexposure to 43°C. (ii) *apaH*(Null) mutants show prolonged synthesis of some heat shock proteins including GroEL, DnaK, and E89 when heat-shocked cells are returned to low temperatures. (iii) *apaH*(Null) mutants exhibit an induction lag when lambda *c*I857(Ts) lysogens are shifted from 30 to 43°C. (iv) Although the level of phosphorylated DnaK increases upon heat shock in wild-type cells, the level in *apaH*(Null) mutants is already high at 30°C and does not increase after a heat shock. (v) Overproduction of DnaK decreases the heat sensitivity of an *apaH ksgA* double mutant and, as previously shown (142), DnaK overproduction suppresses the filamentation phenotype of the double mutant. Together with the observations that DnaK is involved in controlling the stability of σ^{32} (220) and that DnaK protein autophosphorylates (247), these results point to the possibility that AppppN levels affect DnaK functions, thereby modulating the heat shock response. How AppppN affects DnaK functions is not clear at present, although photocrosslinking experiments with N^8-azido-[^{32}P]AppppA show that several stress proteins, including DnaK, GroEL, E89, C45, and C40 are AppppA-binding proteins (126a).

Summary

Bacteria respond to oxidative stress by invoking two distinct stress responses, the peroxide stimulon and the superoxide stimulon (Fig. 2). The two stimulons each contain a set of more than 30 proteins which overlap slightly. The expression of a subset of genes in each stimulon is controlled by a unique regulatory element. These genes constitute the OxyR and SoxRS regulons (Fig. 2). No overlap has been detected between the two regulons, with the possible exception of the *sodA* gene in *S. typhimurium*. The signal for the OxyR regulon induction may be H_2O_2 itself, whereas the signal for the SoxRS regulon is not known. The nature of the proteins outside the regulons and the regulation of the genes encoding these proteins have not been elucidated.

H_2O_2 and O_2^- generators also induce some heat shock

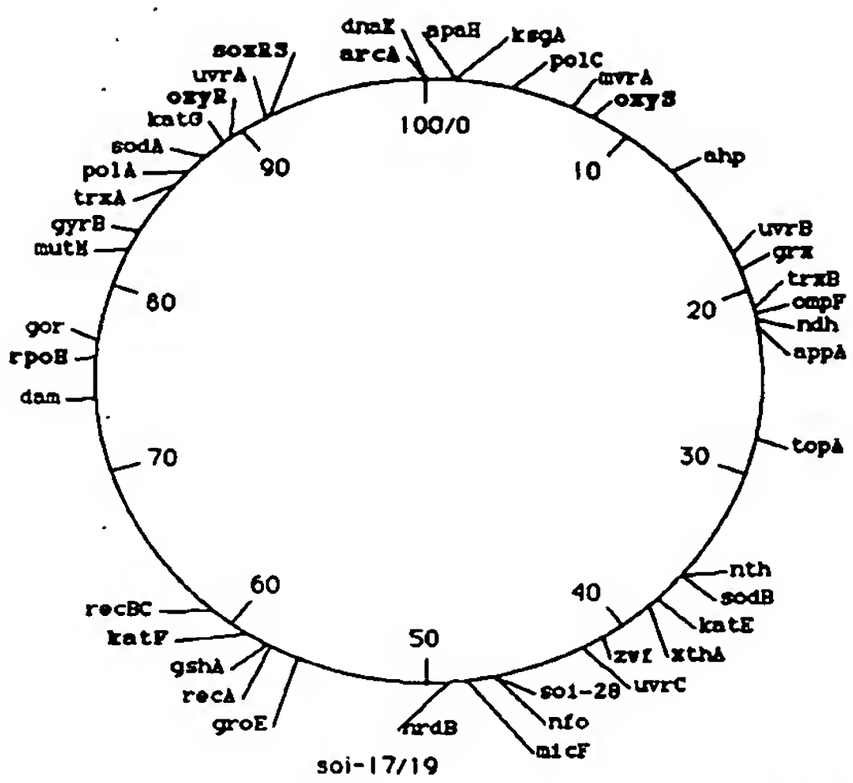

FIG. 3. Map positions of *E. coli* oxidative stress-related genes. The map positions of the genes listed in Table 2 are those of Bachmann (12). The genes in boldface letters are regulatory loci. See the text for details.

proteins, indicating definite overlaps between the heat shock response and the two oxidative stress responses (Fig. 2). Evidence indicates an overlap of the H_2O_2 stimulon with the SOS response. However, the overlap between the superoxide stimulon and SOS response is uncertain. At least three known genes including *xthA* and *katE* are regulated by a sigma factor, KatF (RpoS), whose synthesis is turned on during the stationary phase (Fig. 2). XthA and KatE are known to play important roles in the defense against oxidative stress. However, these KatF regulon genes are not induced by oxidative stress.

Figure 3 shows the locations of the genes that are involved in oxidative stress responses (as described above; Table 1), as well as those that are known to participate in the defense against oxidative stress (see below; Table 2). Although many additional genes are yet to be identified and characterized, the sheer number of genes identified thus far indicates the complexity of the defense systems. The map suggests some tendency toward clustering of the genes. This clustering and the pattern of the cluster locations may reflect the evolution of the *E. coli* chromosome (55, 190). The functional significance, if any, of the clustering is not apparent.

PHYSIOLOGICAL ROLE OF RESPONSES IN OXIDATIVE STRESS

Up to this point, we have discussed genes and proteins induced by oxidative stress only in the context of their regulation. It is equally important, if not, in some cases, equally difficult, to ascribe to them a physiological function vis-à-vis oxidative stress. At the onset, we admit to a teleological bias, namely, that all of the genes and proteins induced by oxidative stress serve a beneficial function. There are examples of proteins, however, whose expression during oxidative stress would appear more detrimental than beneficial, given what is known about their activities.

The defenses against deleterious effects of active oxygen can be logically divided into two broad classes, preventive and reparative. The former class serves to prevent the occurrence of oxidative damage by destroying the offending oxygen species or by limiting the length of certain reactions such as lipid peroxidation and one-electron redox- cycling of quinones. The latter class serves to repair damage caused by offending species that escaped elimination by the prophylactic defense system. Some enzymes, such as alkylhydroperoxide reductase, may serve both roles.

Prevention of Oxidative Damage

Cellular defenses against the damaging effects of oxidative stress involve both enzymatic and nonenzymatic components. The enzymatic components may directly scavenge active oxygen species or may act by producing the nonenzymatic antioxidants. The protective enzymes are, with rare exception, ubiquitous among aerobic organisms. There are four enzymes that provide the bulk of protection against deleterious reactions involving active oxygen in bacteria: SODs (encoded by *sodA* and *sodB*), catalases (*katE* and *katG*), glutathione synthetase (*gshAB*), and glutathione reductase (*gor*). Although some bacteria have NADH-dependent peroxidases specific for H_2O_2, *E. coli* and *S. typhimurium* do not; nor do they have glutathione peroxidase activities.

The reaction catalyzed by SOD is thought to occur in a two-step reaction as follows:

$$Enz_{oxidized} + O_2^- \longrightarrow Enz_{reduced} + O_2$$

$$Enz_{reduced} + O_2^- + 2H^+ \longrightarrow Enz_{oxidized} + H_2O_2$$

There are three types of SOD based upon the metal ligand(s) bound, CuZnSOD, FeSOD, and MnSOD. Each of the metals in the SOD isozymes is a transition metal that facilitates electron transfer. FeSOD is found primarily in prokaryotes. MnSOD is found in both prokaryotes and eukaryotes. CuZn SOD is generally not found in bacteria. One exception is the marine bacterium *Photobacter leiognathi* (210). The second-order rate constants are quite similar, at about 6×10^8 M^{-1} s^{-1}. The steady-state concentration of SOD in *E. coli* is approximately 10^{-5} M (77). The reaction between SODs and O_2^- is first order with respect to O_2^-. The steady-state concentration of O_2^- in a wild-type aerobically growing *E. coli* cell is about 10^{-9} to 10^{-10} M. In *sodA sodB* mutant cells lacking SOD activity, the calculated steady-state concentration of O_2^- is about 5×10^{-6} M (116). Thus the presence of SOD in the cell reduces the steady-state concentration of O_2^- by up to three orders of magnitude.

One of the products of O_2^- dismutation is H_2O_2, which is itself a reactive species. The function of SOD would not appear, therefore, to benefit the cell, unless (i) O_2^- is more toxic than H_2O_2, or (ii) H_2O_2 is disproportionated very rapidly, or (iii) SOD directs O_2^- to H_2O_2 versus other potentially toxic routes of clearance such as through the glutathione radical ($GS^{\cdot}$). Although it is difficult to draw firm conclusions about the relative toxicities of H_2O_2 and O_2^- in vivo, it is certainly true that cellular catalases destroy H_2O_2 with remarkable rapidity. The turnover number for typical catalase is about 10^9 molecules of H_2O_2 disproportionated per active site per second (at 1 M H_2O_2) (236). The electron source is from H_2O_2 itself, and so the reaction is a disproportionation and does not require an exogenous reducing source. Likewise, the reaction is exothermic and does not require ATP. Catalases therefore provide protection against H_2O_2 even in an energy-depleted cell. The two catalases in *E. coli* are found in different cellular locations. HPI and HPII are found in the periplasm and cytoplasm, respectively (110).

This differential localization suggests that sources of H_2O_2 may vary during starvation-dependent and starvation-independent oxidative stress.

Peroxidases are also capable of destroying H_2O_2. Peroxidases, unlike catalase, require NADH or NADPH as an electron source. Under conditions where reducing power is limited, the protective role of peroxidase is likely to be small.

Glutathione (GSH) is an important antioxidant and is synthesized by glutathione synthetase. The high steady-state levels of glutathione in *E. coli* (and presumably in *S. typhimurium*) maintain a strong reducing environment in the cell (150). GSH will react with H_2O_2, O_2^-, or $HOO^\cdot$ to form a stable glutathione radical ($GS^\cdot$). These radicals will then dimerize. Finally, glutathione reductase will transfer an electron from NADPH to the oxidized glutathione (GSSG), to re-form the reduced GSH (165, 205). The overall reaction is shown below.

$$GSH + HOO^\cdot \longrightarrow GS^\cdot + H_2O_2$$

$$GS^\cdot + GS^\cdot \longrightarrow GSSG$$

$$GSSG + 2NADPH \longrightarrow 2GSH + 2NADP^+$$

One of the most important functions of GSH is to reduce disulfide bridges caused by oxidative stress in proteins. Although formation of disulfide bonds is easily reversible, their presence can drastically alter protein function. V_{max} and K_m are modified in many proteins as a function of the thiol/disulfide status (94, 144). Intracellular proteins exist primarily in the thiol state and have a low cysteine content (approximately 2%). Surprisingly, GSH is not essential for maintaining a relatively reduced state of most intracellular proteins, and mutants unable to synthesize GSH (*gshA*) show no increased sensitivity to H_2O_2 and only slight sensitivity to redox active compounds (55a, 89). Other low-molecular-weight thiols including thioredoxin and glutaredoxin may compensate for the lack of GSH (discussed below).

Another enzyme that may play an important protective function is Ahp. This enzyme will reduce many organic hydroperoxides in vitro, including cumene hydroperoxide and *t*-butyl hydroperoxide (124). Mutants lacking Ahp activity are sensitive to killing by these agents (212), and therefore these compounds may be substrates for this enzyme in vivo as well. In addition, a mutant overproducing *ahp* was found to suppress the H_2O_2 sensitivity of *E. coli* Δ*oxyR* cells (90), suggesting that Ahp may act on H_2O_2 directly.

These are the main enzymatic defenses against active oxygen species. It is important to note that peroxidases and the GSH system require a source of reducing power in order to function. In addition, at least in *E. coli*, the most important catalase, KatG, is inducible, as is MnSOD. As we shall see in a later section, reducing power and the ability to synthesize new gene products are limited under conditions of severe oxidative stress. The threshold level of oxidative toxicity may be at the concentration of active oxygen at which the cell can no longer induce or maintain an effective enzymatic defense.

In addition to the enzymatic defenses against active oxygen, most cells employ a wide variety of nonenzymatic organic antioxidants. The main nonenzymatic antioxidants in *E. coli* are GSH and thioredoxin (encoded by *trxA*) (155, 165). Ubiquinone and menaquinone may also serve as membrane-associated antioxidants.

Oxidative Damage and Repair

Oxygen radicals cause a great deal of damage to macromolecules in vitro and in vivo. The oxidative damage that leads to cell death, however, is not always clear. Treatment of cells with redox-active drugs causes both DNA and membrane damage, either of which can be lethal. Oxygen radicals also damage RNA and proteins in vivo. Although damage to RNA and proteins is not likely to be the cause of oxygen radical-induced death, such damage must, at the very least, waste cell energy and resources since the damaged molecule must be either repaired or degraded and replaced. In this section, we will present the major types of damage caused by active oxygen species in vivo. We will also discuss the repair mechanisms that ameliorate that damage.

DNA damage. Cell exposure to H_2O_2, O_2^--generating compounds, ionizing radiation, organic hydroperoxides, singlet oxygen, and ozone results in numerous types of DNA lesions. Both the base and the sugar moieties of DNA are attacked by active oxygen species. Attack on the base produces 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine, and adenine ring-opened and ring-saturated products (for reviews, see references 7, 119, 147, and 197).

Hydroxyl radical attacks on the sugar moiety of DNA lead to sugar fragmentation and production of strand breaks with 3′-phosphate or 3′-phosphoglycolate termini (231). Thymine residues in DNA can be hydroxylated to produce 5-hydroxymethyluracil or oxidatively degraded to produce thymine glycol or a urea residue (31, 60, 133). Guanosine can also be oxidatively degraded in a photooxidative process (215). Addition reactions of $OH^\cdot$ with guanosine residues form 8-hydroxydeoxyguanosine.

In addition to the DNA damage caused directly by oxygen radicals, intermediate organic radicals that are formed during the propagation step of lipid peroxidation can react with DNA. For example, in the presence of autoxidizing methyl linoleate, either the oxidizing fatty acid transfers free radicals to purines (abstracts an H^+), resulting in decomposition of the purines, or the fatty acid radical adds to the purine to form a bulky adduct (200, 227). Incubation of DNA with linoleic acid hydroperoxide also causes site-specific cleavage of double-stranded DNA adjacent to guanidylate residues (200, 227).

Lipid peroxides formed during the initiation and propagation steps decompose into a number of nonradical, stable end products containing a wide range of functional oxygen groups. These include an array of aldehyde, epoxide, hydroxy, carboxy, and peroxy groups, as well as alkanes and alkenes (for reviews, see references 16 and 129). Many stable termination products, such as 4-hydroxyalkenals, epoxides, and other aldehydes have been shown to be directly reactive with DNA, either by alkylating bases (203) or by forming intrastrand and interstrand cross-links (214).

To what extent do these specific reactions occur, and what is their relative significance in vivo? Biochemical analyses of damaged DNA derived from treated cells, coupled with genetic experiments with known DNA repair-deficient mutants (Table 2), allow for an approximate answer to these questions.

Strand breaks and other lesions that block replication are likely to contribute more toward lethality than does base damage that does not hinder replication, although the latter may contribute significantly to mutagenesis. Thymine glycols and 8-hydroxydeoxyguanosine residues are two of the

TABLE 2. *E. coli* genes involved in defense against oxidative stress

Gene	Map position (min)	Regulon	Gene product or activity	Sensitivity to[a]: H_2O_2	Sensitivity to[a]: O_2^-	References
ahpCF	13	OxyR	Ahp	?	?	90, 212
apaH	1		Dinucleotide tetraphosphate hydrolase	+	+	69
appA	22	KatF	Acid phosphatase	?	?	138, 225
arcA	100		Resistance to methylene blue	?	?	217
dam	74		DNA adenine methylase	+	?	242
dnaK	0	HS	Heat shock protein	−	−	132a, 171
gor	77	OxyR	Glutathione reductase	?	?	44
groELS	57	HS	Heat shock proteins	−	−	132a
grx	19		Glutaredoxin	?	?	134
gshA	58		Glutathione synthetase	−	+	88a, 89
gyrB	83		DNA gyrase β subunit	?	+	233a
katE	38	KatF	Catalase HPII	+	−	151, 193
katF	59		σ factor (RpoS)	+	−	138, 153, 225
katG	89	OxyR	Catalase HPI	+	−	154
ksgA	1		16S rRNA methylase	+	+	126b
micF	48	SoxRS	Inhibitor of OmpF translation	?	?	55a, 92
mutM (fpg)	82		Formamidopyrimidine DNA glycosylase	−	?	23, 24, 50
mvrA	7		?	?	+	172
ndh	22	SoxRS (?)	Respiratory NADH dehydrogenase	+	?	118, 119
nfo	47	SoxRS	Endo IV	−[b]	−[b]	48
nrdB	49		Ribonucleoside diphosphate reductase	+	+	66a, 183
nth	36		Endo III	−	−	49
ompF	21		Porin	?	−	55a, 92
oxyR	89		OxyR regulon activator	+	+	44, 90
oxyS	8		General antioxidant/regulator	?	?	125
polA	87		DNA polymerase I	+	?	6
polC	4		DNA polymerase III	+	?	99
recA	58	SOS	Recombination/SOS regulator	+	−	39, 71, 118
recBC	61		Exonuclease V	+	?	117
rimK	?	SoxRS	S6 glutamic acid transferase	?	?	92, 127
rpoH	76		σ^{32} protein	+	+	132a
sodA	88	SoxRS	MnSOD	+	+	37
sodB	36		FeSOD	+/−	+	37
soi-17/19	45–61	SoxRS	?	−	+/−	132
soi-28	47	SoxRS	?	−	+	132
soxRS	92	SoxRS	Regulon activators	−	+	92, 226, 241
topA	28		DNA topoisomerase I	?	?	243
trxA	86		Thioredoxin	?	?	155
trxB	21		Thioredoxin reductase	?	?	155
uvrA	92	SOS	Excinuclease	−	?	50, 146, 196
uvrB	18	SOS	Excinuclease subunit	−	?	50, 146, 196
uvrC	42		Excinuclease subunit	−	?	50, 146, 196
xthA	38	KatF	Exo III	+	+/−	58, 193
zwf	41	SoxRS	Glucose-6-P dehydrogenase	?	?	91, 192

[a] Symbols: +, a mutation in the gene renders cells sensitive; −, not sensitive; ?, not known.
[b] Generally not sensitive, but sensitive to *tert*-butyl hydroperoxide and bleomycin.

most important predominant stable products of oxygen radical attack on DNA (60, 64). Thymine glycol has been shown to block replication in vitro (115). It will also spontaneously decompose to form formylpyruvylurea, urea, and N-substituted urea residues (31). Thymine glycol and its decomposition products are readily removed by an *N*-glycosylase activity associated with endonuclease III (Endo III) (49, 235). Mutants lacking Endo III activity have no detectable thymine glycol glycosylase activity. It is informative that Endo III deficient (*nth*) mutants show no increased sensitivity to killing by H_2O_2 or γ radiation, although *nth* mutations have a mutator effect (31, 117). These observations strongly suggest that thymine glycols are an important premutagenic lesion, but not necessarily a lethal lesion. Consistent with the finding that thymine residue damage is a significant component of general oxidative DNA damage, the bulk of the increased mutagenesis observed in *oxyR* deletion mutants was found to be A · T → G · C transitions (211). A · T base pairs have been shown to be very sensitive to transitions by many chemical oxidants (143).

H_2O_2 causes strand breaks in vivo in the presence of Fe^{2+} (117). Strand breaks also accumulate in H_2O_2-treated cells (6, 71, 100). Single-strand breaks induce the SOS response (148, 233), whereas thymine glycols apparently do not, since *nth* mutants, which lack thymine glycol glycosylase activity, do not show enhanced induction of the SOS response by H_2O_2 (84). In *E. coli*, killing by H_2O_2 is bimodal. The first mode (mode I) is maximal at 1 to 2 mM H_2O_2, is approximately zero order with respect to H_2O_2 concentration, and requires active metabolism. The second mode (mode II) appears to be first order with respect to H_2O_2 between 10 and 100 mM and does not require active metabolism (117). The SOS response is induced by H_2O_2 in proportion to the degree of killing at mode I concentrations (118). Further-

more, *recA* mutants are very sensitive to killing by H_2O_2 (39), and it is the RecA protein itself that is important in cell survival upon exposure to H_2O_2 (118). This suggests that it is the role of RecA in the recombinational repair pathway that is crucial for cell survival against peroxide stress. This conclusion is supported by the observation that *recB* mutants are hypersensitive to H_2O_2, and *sbcB* mutations which activate the RecF pathway of recombination suppress the hypersensitivity of *recBC* mutants to H_2O_2 (117).

The results cited above strongly suggest that the genotoxic damage induced by H_2O_2 is single-strand breaks. That *xthA* (Exo III) mutants are also hypersensitive to killing by H_2O_2 suggests that sugar fragments (blocking groups) must occur with high frequency at the 3′ end of the strand breaks. This has been confirmed in experiments showing that H_2O_2-nicked DNA cannot serve as primers for in vitro DNA synthesis unless incubated in the presence of Exo III (58, 59, 119).

Endo IV, the *nfo* gene product, like Exo III, also removes 3′-blocking groups (48, 59), and overexpression of Endo IV alleviates some of the H_2O_2 sensitivity in an *xthA* mutant (188a). There are differences between Exo III and Endo IV that are discussed below.

Excinuclease (A)BC (encoded by *uvrA*, *uvrB*, and *uvrC*), which plays a crucial role in repair of UV-damaged DNA, was considered to play only a minor role in repair of oxidative DNA damage because *uvrA*, *uvrB*, and *uvrC* mutants are not sensitive to ionizing radiation (114) or H_2O_2 (118). Saporito et al. (196) reported that a *uvrA nfo xthA* triple mutant cannot be constructed, although mutants with any combination of two mutations are viable. Similar results were reported by Goerlich et al. (84). These results suggest a functional redundancy of excinuclease (A)BC, Endo IV, and Exo III. In support of this suggestion, it has been demonstrated that excinuclease (A)BC is capable of removing thymine glycols and AP sites from oxidatively damaged DNA (146). Therefore, it is likely that excinuclease (A)BC also participates in repair of oxidatively damaged DNA. However, the results do not necessarily indicate overlapping specificities of the enzymes. An alternative explanation has been offered by Foster (76a), who showed that relieving the burden of AP sites by using an *ung* mutation allows construction of a *uvrA nfo xthA* triple mutant.

The involvement of excinuclease (A)BC in the repair of oxidative damage is further demonstrated by more recent experiments, which also indicate the participation of the Fpg protein, a DNA glycosylase which has the ability to excise the imidazole ring-opened form of purines. The photosensitizer methylene blue plus visible light generates singlet oxygen as the ultimate reactive species (68) and produces the damaged base 8-hydroxyguanine (202). It has been shown that pBR322 DNA treated with methylene blue plus light transforms *uvrA fpg* double mutants at a significantly lower frequency than it transforms the wild type, whereas the *uvrA* or *fpg* single mutation does not affect the transformation frequency (50). *fpg* mutants are defective in the formamidopyrimidine-DNA glycosylase activity (Fpg protein) (23, 24). The results indicate that both excinuclease (A)BC and Fpg protein are capable of excising damaged bases (perhaps 8-hydroxyguanine and other modified bases) and that these excision activities are a functional duplication for the damage repair.

Finally, H_2O_2 has been shown to stimulate ATP- and Mg^{2+}-independent DNA repair synthesis in vitro (100). As shown with *recA* and *xthA* mutants, *polA* mutants have also been found to be hypersensitive to killing by H_2O_2 (6). Taken together, these findings show that DNA repair synthesis after H_2O_2 damage requires DNA polymerase I activity. Furthermore, *polC*(Ts) mutations encoding thermolabile DNA polymerase III sensitize cells to H_2O_2 at 43°C and *polC*(Ts) mutants fail to repair single-strand breaks caused by H_2O_2 when incubated at the high temperature (99). Therefore, DNA polymerase III is also required for repair of DNA damage by H_2O_2. It has been proposed that at least two pathways are involved for repair of H_2O_2-induced DNA damage: one requires Exo III and DNA polymerases I and III, whereas the other utilizes DNA polymerase I (101).

Mutagenicity. Since oxygen radicals cause DNA damage in vitro and in vivo, and since DNA repair-deficient mutants are hypersensitive to oxidative stress, it is not surprising that oxidative stress leads to mutagenesis. It has been known since the mid-1950s that hyperbaric oxygen is mutagenic in *E. coli* (73). Even concentrations of oxygen physiological to humans have been reported to be mutagenic in *S. typhimurium* (35), although not so in *E. coli* (70).

The mutagenic potential and pathway for H_2O_2 has been fairly well established (38, 118). As with killing, H_2O_2 mutagenicity is biphasic. At low (mode I) concentrations, mutagenicity is strongly correlated with SOS induction as measured by induction of a lambda lysogen (118). At high (mode II) concentrations, the correlation is weaker. The SOS-dependent mutagenesis observed after UV irradiation depends largely upon expression of the *umuDC* operon (13, 15). Surprisingly, H_2O_2 mutagenesis was reported to be UmuDC independent in *E. coli* (118). And yet, *lexA*(Ind$^-$) mutants are even more sensitive to killing by H_2O_2 than are *recA* mutants, suggesting that other SOS functions are important for cell survival (118). Furthermore, as mentioned above, H_2O_2 induces cell filamentation in an *sfiA*$^+$-independent manner (118). The role of the SOS response in H_2O_2 killing and mutagenicity is therefore somewhat unusual.

Another approach to understanding the mutagenic potential and specificity of H_2O_2 was taken by Storz et al. (211), who used *oxyR*Δ mutants in an *S. typhimurium* His$^-$ → His$^+$ reversion assay. When *oxyR*Δ mutants are grown under normal oxygen tension, they show a His$^-$ → His$^+$ reversion frequency 11-fold higher than do isogenic wild-type cells. Interestingly, the mutagenesis in an *oxyR*Δ strain carrying *mucA*$^+$ and *mucB*$^+$ on a plasmid (pKM101) is 50-fold higher than in the wild-type with the same plasmid. These results suggest that the products of *mucA*$^+$ and *mucB*$^+$ enhance mutagenesis by converting premutagenic lesions to mutagenic lesions. Since *mucA* and *mucB* are plasmid-encoded analogs of the *E. coli umuDC* genes, this finding indicates either that the paths of H_2O_2 mutagenicity differ in *S. typhimurium* and *E. coli* or that there is an unresolved discrepancy in the results. The difference in results may simply reflect a difference in mutagenicity markers. The *oxyR* and pKM101 mutational analysis involved a His reversion assay (211), whereas Imlay and Linn (117, 118) used forward mutation markers.

As noted above, the most frequent type of mutation in the *oxyR*Δ strain is A · T → G · C transitions, as might be expected if oxidative damage to thymine residues is prevalent. Similar results were obtained with *E. coli* or *S. typhimurium* cells lacking catalase activity or Ahp activity (211).

The nature and pathway of O_2^- mutagenicity are less clear. Since O_2^- is not very reactive toward many biological substrates in vitro (18–20), there remains the important question of how dangerous an increase in the flux of O_2^- is in vivo and whether endogenously produced O_2^- poses a threat to genetic integrity. Some redox-active compounds

have been shown to be slightly mutagenic (43, 71, 170). Given the pleiotropic effect of many redox compounds, however, the results did not exclude the possibility of O_2^--independent mutagenicity of these compounds. In fact, O_2^- that is generated by the hypoxanthine-xanthine oxidase system has been shown to cause a significant increase in mutations only in a particular strain (TA104) of *S. typhimurium* which carries a *uvrB* mutation (54). The possible involvement of excinuclease (A)BC in oxidative DNA damage repair has been discussed above.

By using a different approach, both the mutation frequency and rate in SOD^- cells (i.e., *sodA sodB* double mutants) have been determined in the absence of redox-active compounds (70), when the steady-state concentration of O_2^- is ca. 10^3 times higher than in wild-type cells (116). The mutation rate was determined by a fluctuation test measuring the rate of rifampin-sensitive to rifampin-resistant mutagenesis during aerobic growth of SOD^- cells. The target of the antibiotic rifampin is the β subunit of RNA polymerase, and resistant mutants possess an altered subunit as a result of mutations in the *rpoB* gene. Such mutations must be mostly, if not exclusively, base substitutions (83). The mutation rate toward rifampin resistance is thus a sensitive assay for this class of mutational events. The *sodA sodB* double mutant shows a 40-fold increase in the rate of aerobic spontaneous mutations toward rifampin resistance, and the *sodA* single mutant shows a 9-fold increase compared with the wild type; the *sodB* single mutant do not show an increase in the mutation rate (70, 223).

The increased mutagenesis observed in SOD-less cells is oxygen dependent. In anaerobic cultures, the frequency of $Thy^+ \rightarrow Thy^-$ mutations is essentially identical for the *sodA sodB* double mutant and the wild-type strain. When SOD-less cells are subjected to bubbling of pure oxygen through the culture or are exposed to plumbagin, they display a very high mutation frequency. These results indicate that the increase in spontaneous mutagenesis in cells lacking SOD activity depends on the presence of oxygen and that exposure to increased levels of O_2^- greatly enhances mutagenesis.

In keeping with the finding that O_2^--generating agents do not induce the SOS response (71), the presence of a *recA*(Def) mutation does not alter the oxygen-dependent mutagenesis enhancement in *sodA sodB* mutants (70). Furthermore, by using *dinD*::*lacZ* and *sfiA*::*lacZ* fusions (*dinD* and *sfiA* genes are repressed by LexA), it has been shown that SOD-less cells do not display induction of the SOS response. Thus, unlike H_2O_2 mutagenesis, O_2^- mutagenesis appears to be completely independent of the SOS response.

Exo III, encoded by the *xthA* gene, has several catalytic activities that are involved in the repair of certain types of DNA damage. The enzyme possesses 3′ exonuclease and phosphatase activities, RNase H, and apurinic endonucleolytic activities, as well as an endonucleolytic activity that recognizes bases containing urea residues (133, 168, 238). *xthA* mutants lack 90% of the normal Exo III activities (238). When mutation frequencies in wild-type, *sodA sodB*, and *sodA sodB xthA* strains were measured, it was found that the enhanced mutation frequency observed in the *sodA sodB* culture is largely dependent on the presence of a functional *xthA* gene (70, 113). The observation suggests that cells lacking SOD suffer from increased levels of premutagenic DNA damage and that the action of Exo III on these DNA lesions greatly enhances their mutagenicity.

Little is known about the mutagenic specificity of O_2^--inflicted DNA damage. Evidence shows that it cannot be identical to that induced by H_2O_2. First, it is SOS independent. Second, whereas SOD-less cells show higher plating efficiency of O_2^--damaged λ damage than wild-type cells do, they show no difference in plating efficiency of H_2O_2-damaged phage (71a). Finally, O_2^- induces synthesis of Endo IV, whereas H_2O_2 does not. It is probable that Endo IV and Exo III do not have identical substrate specificities since overproduction of Endo IV in an *xthA* mutant does not complement the H_2O_2 sensitivity defect of the mutant (48). Also, *nfo* mutants are sensitive to bleomycin and cumene hydroperoxide, whereas *xthA* mutants are not sensitive to bleomycin (48).

A · T base pairs are the major targets for substitutions in *E. coli* mutants lacking SOD activity (131a). Furthermore, both Endo III and MutY adenine glycosylase (specific for A-G mismatches) may be $(Fe\text{-}S)_4$-containing proteins (167). Several $(Fe\text{-}S)_4$-containing proteins have been shown to be remarkably sensitive to inactivation by O_2^- (80). It is therefore possible that the elevated mutagenesis in SOD-less cells comes from inactivation of one or both of these repair enzymes. *mutY* mutants show a high frequency of transversions (9), whereas H_2O_2 mutagenesis largely generates transitions. If determination of the mutagenic specificity in SOD-less cells turns up an elevated frequency of transversions, this might suggest a role of MutY in O_2^--dependent mutagenesis.

Since elevated concentrations of oxygen are mutagenic in *E. coli*, it was of interest to determine the role, if any, of the SOS response in that mutagenesis. The SOS response includes error-prone repair functions that create mutations at the site of DNA lesions (targeted mutagenesis), as well as functions that mutate undamaged DNA ("untargeted" mutagenesis) (239). It is possible, however, that all DNA contains cryptic lesions, revealed only when fixed as mutations in an SOS-induced cell. Active oxygen species are a potential source of such lesions. To determine whether untargeted SOS mutagenesis is oxygen dependent, the frequency of $His^- \rightarrow His^+$ reversions in a *recA441*(Tif) strain was compared when cells were grown aerobically or anaerobically. The *recA441*(Tif) mutation allows activation of RecA protein at 42°C without DNA damage, leading to an increase in the spontaneous mutation rate. The results revealed no reduction in Tif-mediated untargeted mutagenesis in the absence of oxygen. It was concluded that untargeted SOS mutagenesis is not responding to oxygen-dependent lesions (70).

Although ozone and singlet oxygen have both been shown to cause DNA damage in vivo and in vitro, their roles in oxidative mutagenesis remain controversial (51, 63, 68, 145, 199, 230).

DNA damage and transcription. As discussed above, thymine glycol and possibly other damaged bases, as well as AP sites, block replication by DNA polymerase. It has recently been shown that thymine glycols also interfere with transcription by causing RNA polymerase to pause or stop completely at or near the lesion (35a). Although other forms of oxidative DNA damage have not been tested, it is probable that single-strand breaks, AP sites, 8-hydroxydeoxyguanosine, and many other forms of oxidatively damaged DNA either block RNA polymerase or cause misreading. Both of these effects would result in production of truncated or abnormal proteins, a result that, if severe enough, would itself induce the heat shock response (85). The chaperone proteins, DnaK and GroEL, show increased synthesis after oxidative stress, suggesting the possibility that they serve to handle the increased level of misfolded proteins (discussed

in detail below). Toxicity induced by severe oxidative DNA damage may result directly from disruption of transcription as well as from mutagenesis.

Membrane damage. Oxidative damage to membranes can arise through either lipid or membrane protein damage. Oxidative stress has been shown to cause peroxidation of lipids both in vitro and in vivo. Of the active oxygen species formed during oxidative stress, $HOO^{\cdot}$, $OH^{\cdot}$, singlet oxygen, ozone, and perferryl radicals can all initiate lipid peroxidation in vitro and in vivo. Lipid peroxidation entails three steps: initiation, propagation, and termination. It is likely that a lipid peroxidation chain reaction begins after hydrogen abstraction from an unsaturated fatty acid to form a lipid radical. The lipid radical ($L^{\cdot}$) thus formed reacts with molecular oxygen to form a lipid peroxy radical ($ROO^{\cdot}$). The reaction is perpetuated when the lipid peroxy radical attacks another unsaturated fatty acid and abstracts a hydrogen atom to form a fatty acid hydroperoxide (ROOH) and perpetuate the initial reaction. The hydroperoxides thus formed will break down thermally or in the presence of O_2^- or reduced transition metals to form lipid peroxy radicals ($LOO^{\cdot}$) or lipid alkoxy radicals ($LO^{\cdot}$), both of which can initiate new rounds of peroxidation. It is also possible that NADH will donate an electron to the hydroperoxide to generate another lipid radical and water (129).

Lipid alkoxy radicals can undergo cleavage of C—C bonds to form unsaturated fatty acid aldehydes and alkyl radicals. The peroxidation of lipids thus generates products which are shorter than the initial fatty acid. In addition to producing fatty acyl chains that are shorter than the parent chain, the end products of lipid peroxidation include alkanes, ketones, epoxides, and aldehydes (16, 129, 163). When fatty acid chains become shortened or gain charges, their ability to rotate within the membrane is altered and the membrane becomes more fluid (162, 163). An increase in membrane fluidity results directly in a loss of structural integrity. Structural integrity is required for transport of most nutrients, F_1/F_0 ATPase activity, motility, and prevention of osmotic imbalance (62, 130, 140). Because membrane permeabilization will destroy the proton gradient across the cell membrane, the internal pH will drop, causing the $O_2^- \rightleftharpoons HOO^{\cdot}$ equilibrium to shift to the right, which in turn is likely to result in further oxidative damage (72).

A second hazard posed by lipid peroxidation is that peroxidation intermediates and end products are mutagenic. As stated above, lipid peroxidation products include an array of aldehyde, epoxide, hydroxy, carboxy, and peroxy groups, as well as alkanes and alkenes. Many stable termination products, such as 4-hydroxyalkenals, epoxides, and other aldehydes have been shown to be directly reactive with DNA, either by alkylating bases (203) or by forming intrastrand and interstrand cross-links as discussed above (214). Lipid oxidation products listed above also react with and inactivate proteins (42).

E. coli and *S. typhimurium* are defended against lipid peroxidation in both simple and complex ways. The rate of fatty acid peroxidation is directly proportional to the number of unsaturated C=C bonds (129), and bacteria have generally saturated or monounsaturated fatty acids in their membranes. It has been shown that *E. coli* fatty acid auxotrophs grown on oleic acid (monounsaturated fatty acid) were more sensitive to killing by oxidative stress than were those grown on saturated fatty acids (106); therefore, that there is a cost associated with maintaining even monounsaturated fatty acids in the membrane. Additionally, certain stress conditions such as exposure to ethanol have been shown to cause an increase in the level of unsaturated fatty acids into the membranes (17). Taken together, these data suggest that bacteria are not altogether immune to oxidative lipid damage.

In *E. coli*, there may be an inducible membrane repair response. It has been shown that H_2O_2 disrupts membrane functions at nonlethal doses and that cells which are pretreated with a low concentration of H_2O_2 acquire the ability to rapidly recover from the loss of membrane functions (72). The peroxide stress response is required for this inducible recovery of membrane function. Furthermore, *katG* (under control of *oxyR*) plays a necessary, although not sufficient, role in this response (72). It is possible that the OxyR-regulated Ahp (*ahpC ahpF*) plays an important role in inducible membrane repair by reducing fatty acid hydroperoxides. In these experiments, membrane functions were monitored by measuring ΔpH-dependent and ΔpH-independent transport. The concentrations of H_2O_2 sufficient to induce rapid recovery of transport inhibition has no effect upon transport activities. This would imply that the signal to induce transport recovery does not initiate with damage to the membrane itself.

Other evidence for the existence of an inducible membrane response comes from experiments showing that ionizing radiation induces a membrane repair response as measured by sensitivity of naive and preexposed cells to hypotonic medium (244). Cells treated with chloramphenicol prior to the inducing treatment failed to develop resistance to osmotic stress, suggesting that novel gene products are required for induced resistance.

Protein damage. Interactions between oxygen radicals and proteins leads to conversion of proline and arginine residues into carbonyl derivatives. Oxidative attack of histidinyl and prolyl residues converts them into asperginyl and glutamyl derivatives, respectively (208). Methionine and cysteinyl residues are oxidized to form methionine sulfoxide derivatives and disulfide derivatives, respectively. These alterations generally inactivate enzymes and can lead to their targeted degradation. Several *E. coli* enzymes have been shown to be quite sensitive to oxidative damage, including dihydroxy acid dehydratase, glutamine synthetase, ribosomal protein L12, quinolate synthetase, aconitase, and 6-phosphogluconate dehydratase (32, 80, 135, 144). Dihydroxy acid dehydratase, quinolate synthetase, aconitase, and 6-phosphogluconate dehydratase have $(Fe\text{-}S)_4$ clusters, and it is likely that these are the sites of O_2^- attack (80). It would appear to be generally true that metal-binding sites in proteins are especially sensitive to attack by active oxygen species. It will be of value to determine whether Endo III and MutY are inactivated by O_2^-, since they may contain $(Fe\text{-}S)_4$ clusters.

Repair of protein damage appears to be limited to reduction of disulfides and methionine sulfoxides. Reduction of both of these is facilitated by thioredoxin and thioredoxin reductase in an NADPH-dependent pathway (86, 155). Little is known about the regulation of inducibility of protein repair enzymes.

In *E. coli*, there appear to be specific proteinases that selectively degrade oxidized proteins (52). This degradation pathway is ATP independent and is distinct from the Lon protease associated with the heat shock response (53). A protease has been purified from *E. coli* that selectively degrades oxidized glutamine synthetase (191). Whether this specific degradation serves simply to expedite recycling of amino acids, or whether it has some other physiological function (for example, to prevent accumulation of damaged

proteins that can cause induction of the heat shock response or accumulation of glutamate owing to inactivation of a high proportion of the total glutamine synthetase) is not currently known. Regulation of these proteases is also poorly understood, although OyxR(Con) mutants show a two-fold increase in protease activity, suggesting a possible role for OxyR in their induction (53).

Role of the Peroxide Stress Response

We know the biochemical functions of only a minority of OxyR- and non-OxyR-regulated genes that are induced in the peroxide stress response. The OxyR-regulated genes whose functions are known are *katG* (HPI catalase), *gor* (glutathione reductase) and *ahpC-ahpF* (alkylhydroperoxide reductase). The role of HPI catalase is to reduce the concentration of H_2O_2. The reduction of GSSG by glutathione reductase would be useful for maintaining the reducing environment of the cell and for directly reacting with and destroying H_2O_2. Surprisingly, however, mutant cells completely devoid of GSH (*gshAB* mutants) show no increased sensitivity to H_2O_2 (89), raising the question of the role of glutathione reductase during exposure to peroxide stress. Ahp activity is likely to play two important roles during exposure to peroxides. By reducing the level of the offending peroxide, it diminishes the threat of damage. Also, by reducing the level of peroxidized cellular components, it repairs the damage that is not prevented from occurring in the first place.

Another activity that may be at least partially regulated by OxyR is that of ribonucleoside diphosphate reductase (66a), which converts all ribonucleoside diphosphates to 2′-deoxyribonucleoside diphosphates, precursors for DNA synthesis. Ribonucleoside diphosphate reductase can use either glutaredoxin or thioredoxin as a hydrogen source. GSH is required for synthesis of glutaredoxin. As discussed above, one of the OxyR-regulated genes, *gor*, encodes glutathione reductase. One possible role of enhanced levels of glutathione reductase may be to maintain sufficient levels of GSH so that glutaredoxin levels do not fall dramatically during oxidative stress. H_2O_2 has been shown to inhibit incorporation of radiolabeled thymine into the DNA, and pretreated cells show a more rapid recovery from inhibition of incorporation after a challenge dose than naive cells do (56). Since H_2O_2 treatment also disrupts uptake of thymine (72), it is not clear whether H_2O_2 also directly inhibits DNA synthesis. If it does, however, and recovery of DNA synthesis after H_2O_2 treatment requires OxyR functions, the candidate functions would be ribonucleoside diphosphate reductase and glutathione reductase.

The heat shock proteins DnaK and GroEL are inducible with H_2O_2 (171). GroEL is induced by O_2^- (234). DnaK makes up approximately 1% of the total cellular protein at 37°C and approximately 4% after a temperature shift (182). It possesses several activities in vitro: (i) it is essential for in vitro replication of λ phage, (ii) it has a 5′ nucleotidase activity that is inhibited by AppppA, and (iii) it autophosphorylates (247) in a temperature-dependent manner (159b). *dnaK* mutants also do not phosphorylate glutaminyl- and threonyl-tRNA synthetases (232). Finally, DnaK plays an important role as a chaperone protein (see reference 96 and references therein). Since DnaK is induced by H_2O_2, the presumption is that it must play a role during peroxide stress. Which, if any, of the above functions is important in the response of the cell to peroxide stress is not known. It is interesting that AppppA is strongly induced by H_2O_2, starvation, and heat shock conditions that all induce DnaK. AppppA has recently been shown to bind to DnaK and increase the level of DnaK autophosphorylation in vitro (126a).

GroEL functions as a chaperone that maintains prefolded proteins in that state and facilitates their export through the inner membrane. Increased production of GroEL during oxidative stress may be required to handle an increased number of misfolded proteins resulting directly from damaged nascent polypeptides or indirectly from mistranscribed or mistranslated genes. Alternatively, increased levels of GroEL may be required to compensate for damaged membrane export apparatus.

Finally, H_2O_2 and O_2^- exposure induces two distinct NADH-dependent diaphorase activities as determined by activity bands on nondenaturing protein gels (70c). The role of diaphorase (NADH dehydrogenase) activities during oxidative stress is not clear. One possible function is to reduce the concentration of intracellular NADH in order to prevent the reduction of Fe^{3+} to Fe^{2+} by NADH, as reported previously (97). Consistent with the idea that high NADH concentrations might pose a threat to cells during exposure to H_2O_2, *ndh* mutants (which accumulate NADH) are hypersensitive to killing by H_2O_2 (119). A second function might be to reduce the level of cellular respiration and thereby reduce the number of electrons flowing through the electron transport chain (a source of O_2^-). A third possible function of NADH-diaphorase activity is to transfer electrons to ubiquinone-8, which can then be oxidized in a coupled manner to generate ΔpH and Δψ (see reference 185 and references therein), both of which are lost during oxidative stress (72).

Role of the Superoxide Stress Response

As discussed above, the SoxRS-regulated genes that encode known functions are *sodA* (SOD), *nfo* (Endo IV), *micF* (antisense regulator of *ompF*), *zwf* (glucose-6-P dehydrogenase), and *rimK* (an enzyme that adds nonencoded glutamic acid residues to the carboxy terminus of the ribosomal protein S6) (92, 127). A SoxRS-regulated function whose gene has not been characterized is an NADH diaphorase (70c).

The function of SOD is to reduce the level of O_2^-. Endo IV repairs oxidatively damaged DNA by removing 3′-blocking groups such as phosphoglycolates. As discussed above, there must be some substrate specificities not shared by Endo IV and Exo III. The function of the *micF* antisense RNA in down-regulating *ompF* has been discussed above. *soxR*(Con) mutants show increased resistance to several antibiotics whose uptake may be OmpF dependent, and it has been hypothesized that the natural function of OmpF down-regulation by SoxR is to exclude naturally occurring antibiotics (92). Another possible function of OmpF down-regulation might be to limit the uptake of naturally occurring redox-active compounds that promote O_2^- production. However, *ompF* mutants are not more resistant to menadione than is the wild type (55a).

The role of glucose-6-P dehydrogenase is to produce NADPH. NADPH production would be useful under conditions of oxidative stress since it is required as the electron source for both thioredoxin reductase and glutathione reductase. Also, protein repair catalyzed by methionine sulfoxide reductase requires NADPH. Furthermore, NADPH does not reduce Fe^{3+}, whereas NADH does (146a). Thus NADPH production would provide the cell with reducing

equivalents without facilitating $OH^{\cdot}$ production via the Fenton reaction.

The role(s) for the NADH-dependent diaphorase activity induced by O_2^- stress is likely to be the same as that of the H_2O_2-induced diaphorase, namely, to reduce the level of NADH, to decrease the rate of respiration, or to detoxify redox-active quinones to the hydroquinone state by a two-electron reduction. In *E. coli*, one of the NADH dehydrogenases is the product of the *ndh* gene (185). It is not known whether either of the oxidative stress-inducible diaphorases is the product of the *ndh* gene, although upstream sequence homology between the *ndh* gene and several SoxRS-regulated genes suggests that this gene may be regulated, at least in part, by SoxRS (82a). The functions of *soi-17/19* and *soi-28* are not yet known, although these genes must play an important role since they sensitize cells to O_2^- stress (132). The function of *mvrA* is presently unknown.

Possible Role of the Stringent Response

Another response that may play a role in oxidative stress is the stringent response. When uncharged tRNAs enter the A site of a ribosome during translation, the ribosome stalls and a ribosomal protein, the RelA protein, produces a stringent factor, 3',5'-tetraphosphoguanosine (ppGpp). The ppGpp then binds to RNA polymerase and alters the transcription from *rrn* operons (149). The net effect is that during amino acid starvation, the cell decreases or suspends production of rRNA and ribosomal protein. This suspension serves at least one apparent purpose: it saves the cell the costly expenditure of precursors and energy required to synthesize ribosomes when those ribosomes would end up being unemployed owing to the lack of amino acids.

Interestingly, ppGpp is also produced during heat shock and during exposure to H_2O_2 and paraquat (33, 228). Certain quinones and quinonelike drugs that are capable of redox cycling have been shown to inhibit leucyl-tRNA synthetases in vitro, suggesting that in vivo, leucyl-tRNAs would be unloaded in the presence of these compounds, triggering the stringent response (181). In addition, increased levels of ppGpp have been detected in *E. coli* after exposure to hyperbaric oxygen, and it has been proposed that this accounts for the inhibition of protein synthesis observed in these cells (204). In *E. coli*, an enzyme required for branched-chain amino acid biosynthesis (dihydroxy-acid dehydratase) is highly sensitive to inactivation by O_2^- (80), and SOD-less mutants are auxotrophs for these amino acids (37). It is therefore likely that O_2^--mediated stress causes induction of the stringent response by inactivating dihydroxy-acid dehydratase. All of the above inducing stresses are likely to disrupt normal translation (229). Some oxidative stress disrupts translational machinery, the stringent response may serve to limit production of unnecessary stable RNA. It would make sense for the cell to slow general protein synthesis under conditions where the membrane and DNA are under oxidative attack. Indeed, there is preliminary evidence that in *relA* mutants exposed to plumbagin, DNA synthesis decreases while the optical density of the culture increases (70d).

Other Genes Involved in Protection against Oxidative Stress

In addition to the genes and proteins discussed above, several genes are constitutively expressed, and there are genes whose regulation is SoxRS and OxyR independent and whose functions have been demonstrated to be necessary during oxidative stress, based on the fact that mutations in these genes sensitize the cell to oxidative stress. These include, but are not limited to, *apaH* (AppppN hydrolase) (126a) and *ksgA* (16S rRNA methylase) (70b). Mutations in the *xthA* (Exo III), *dam* (DNA adenine methylase), *gyr* (DNA gyrase), and *topA* (topoisomerase I) genes also play a role in protection against the oxidative damage. There are also mutations in genes whose functions are unknown, but nevertheless render the cell more sensitive to oxidative stress. These include *soi-17/19*, *soi-28*, *mvrA*, and several *oxyS* genes (125, 132, 172). In addition, there are several other genes whose functions would strongly suggest that they play an important role in protection against oxidative damage, but whose sensitivity to oxidative stress has not been determined, e.g., *trxA* (thioredoxin). These genes are included in Table 2.

CONCLUSIONS

Most of the proteins induced by either peroxide or O_2^- stress remain unidentified. Consequently we know relatively little about the physiological functions or pathways used during oxidative stress. The few functions that have been identified, however, suggest that many, if not most, of the metabolic pathways in the cell are altered in some way by oxidative stress and the responses of the cell to it. For example, oxidative membrane damage leads to a plethora of subsequent problems for the cell; loss of ΔpH will change the intracellular pH, causing further production of $HOO^{\cdot}$, will decrease ATP synthesis, will increase permeability to toxic compounds, will prevent chemotaxis, etc. Each one of these events requires further metabolic adjustments by the cell. For example, the loss of energy charge is likely to affect macromolecular synthesis, which, in turn, might induce the stringent response, and oxidative DNA or protein damage is likely to generate its own sequelae of events.

One approach to unraveling the complex responses of the cell to oxidative stress may therefore be to determine which types of damage or disruption inevitably follow one another. For example, certain types of DNA damage may generally lead to production of truncated or abnormal proteins. Because of the general cooccurrence of these events, the cell may have evolved a regulon that coinduces a response to deal with abnormal proteins, whether or not abnormal proteins have actually been produced. An example of this might be in the SoxRS response, in which the cell coregulates induction of antioxidants such as MnSOD and a DNA repair enzyme, Endo IV. It is unlikely that at the time of initial activation of the SoxRS regulatory elements, severe DNA damage has already occurred. Rather, through evolutionary experience, the cell may "know" that whenever it needs extra MnSOD, it is likely to need extra Endo IV. In terms of assigning genes or functions to stimulons, coinduction of genes or functions rather than sequential inductions would be suggestive of, although not proof of, coregulated events. Final assignment of genes to stimulons can, however, be made only when a necessary interaction between the regulators (SoxR, SoxS, OxyR, etc.) and the genes they regulate (*nfo*, *soi-28* promoters, etc.) has been demonstrated.

Comparison and Extrapolation to Eukaryotes

There is a great deal of similarity in the overall strategy of defense against oxidative stress between bacteria and eukaryotic cells. On the other hand, there appear to be some

crucial tactical differences. Nearly all aerobic cells, prokaryotic and eukaryotic, possess SODs, catalases, and peroxidases to destroy active oxygen species. Indeed, SOD expressed from mammalian *sod* genes fully protects SOD^- bacteria from O_2^- (175). Repair functions also overlap extensively. For example, some bacterial and eukaryotic DNA repair enzymes specific for oxidative damage share extensive homology (55a, 185a).

The fundamental differences between bacteria and eukaryotes appear in the regulation and complexity of the responses to oxidative stress. The inducers of the oxidative stress responses in bacteria appear to be either the oxidant itself or interaction of the oxidant with a cell component, as in the case of OxyR. On the other hand, oxygen radicals do not, in general, induce limited production of antioxidant enzymes in mammalian cells. Rather, cytokines such as tumor necrosis factor, interleukin-1, or bacterial lipopolysaccharides induce SOD synthesis and multigene responses (157a, 240). Evolutionally this makes sense, given that most mammalian cells exist in an environment where the oxygen concentration is constant (ambient 4% in blood serum). An exception is upon sequestration and activation of neutrophils to produce active oxygen species. It is economical that the same signals that sequester and activate neutrophiles should also induce antioxidants in bystander cells. Indeed, although paraquat has no inductive effects on MnSOD and phase II enzymes in mammalian cells, tumor necrosis factor is a very potent inducer (240).

Sources of active oxygen species of evolutionary importance (that fluctuate sufficiently to warrant inducible responses) include near-UV radiation, ionizing radiation, and possible ingestion of redox-active or radical generating phytoalexins (i.e., psoralen in celery). Mammalian cells induce a few proteins in specific response to NUV stress, most notably heme-oxygenase and glutathione transferase. Since cells of internal organs that are never exposed to near-UV show strong inducibility of these proteins by near-UV (131), this suggests either that their regulation pathway is ancient, or that the actual natural inducer is not near-UV, but rather a subsequent reactant, possibly H_2O_2. The regulation pathway of H_2O_2-inducible genes in mammalian cells is unknown. It is possible, however, that simple activation of a DNA-binding protein results in gene expression, as appears to be the case in bacterial OxyR-regulated genes.

Although the regulatory pathway(s) appears to be enormously complex and involves several factors, recent work shows that O_2^- is a strong tumor promoter that works by activation and induction of growth competence-related gene products; c-*fos*, c-*jun*, c-*myc*, and β-actin (40a, 47). These gene products are transcription factors that regulate a battery of genes. Other factors involved in the regulation of eukaryotic antioxidant gene expression include an induction of calmodulin kinase by increase in Ca^{2+} concentrations, which in turn activates S6 phosphorylation. The elevation of Ca^{2+} concentration levels, which could occur when membranes are damaged by O_2^- radicals, also leads to phosphokinase C activation. Phosphokinase C activation in turn phosphorylates and activates topoisomerase I (184a). Phospholipid hydroperoxides, produced by membrane oxidation, will also activate phospholipid hydroperoxide reductase, glutathione peroxidase, and phospholipase II.

Finally, DNA strand breaks induced by oxidative stress will induce poly(ADP-ribose) polymerase. Several proteins, including Jun, Fos, TopoI, and a DNA polymerase α subunit, are poly-ADP-ribosylated. Poly-ADP-ribosylation is crucial for activation of Fos. The relationship between an increase in the concentration of calcium and DNA damage is unclear. Other factors involved in increased expression of the c-*fos* gene upon oxidative stress include the serum-responsive factor, serum-responsive element, and cyclic AMP-responsive element (40a).

In summary, the complexity in bacterial responses appears to be in the sheer number of proteins induced by oxidative stress, well over 60. Conversely, the relative number of proteins induced in mammalian cells is small, but the regulatory pathways are highly complex.

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Article IV

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Defenses against Oxidative Stress in *Neisseria gonorrhoeae*: a System Tailored for a Challenging Environment

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INTRODUCTION

Neisseria gonorrhoeae (also known as the gonococcus) colonizes primarily the human genitourinary tract, giving rise to the sexually transmitted infection gonorrhea. Disease caused by this organism is a significant health problem despite continual advances in treatment (24, 25, 87, 250). Worldwide there are an estimated 62 million new cases a year, with an average of 22 million cases at any given time (87).

N. gonorrhoeae inhabits mainly mucosal surfaces of the urethra in males and the cervix in females and as a consequence is exposed to a variety of oxidants, which are generated in three main ways: (i) as a by-product of the bacterium's own metabolic processes, (ii) as a key element of the innate immune response, and (iii) as a result of exposure to other factors within the host environment that promote oxidative stress, such as metal ions or commensal organisms that generate oxidants. The most commonly found and discussed oxidants in biological systems are the reactive oxygen species (ROS), which include the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$), and the reactive nitrogen species (RNS), which include nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$). In addition, sulfur- and chlorine-containing compounds are generated by antimicrobial effector cells (103, 125, 227). Oxidative stress causes damage to DNA, proteins, and cell membranes and often results in cell death (reviewed in reference 125).

Mechanisms for coping with oxidative stress are crucial for the survival of all organisms, particularly obligate human pathogens, such as *N. gonorrhoeae*, that are routinely exposed to oxidative killing by the host and inhabit an environment of unremitting oxidative stress. Defenses against oxidative stress are increasingly being recognized as playing an important role in virulence (28, 67, 102, 105, 123, 206, 251, 258). A greater understanding of the oxidative stress response of microbial pathogens may aid the future development of treatment and

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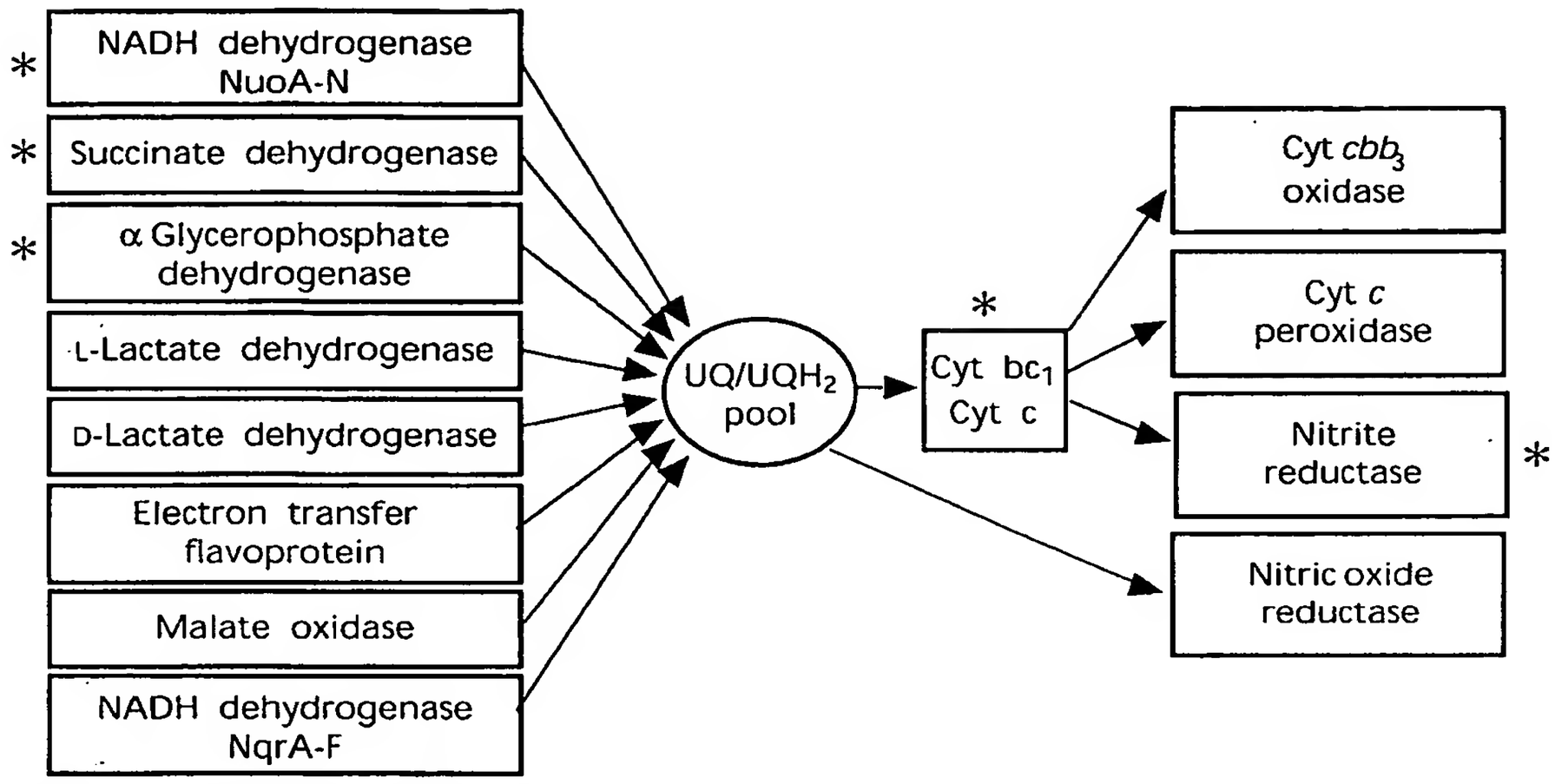

FIG. 1. Electron transport and sources of endogenous ROS and RNS in *N. gonorrhoeae*. Respiratory chains of *N. gonorrhoeae* and *N. meningitidis* are shown, as determined from genomic and biochemical information. Cyt, cytochrome; UQ, ubiquinone. Asterisks indicate potential sites of oxidant generation.

prevention strategies for disease caused by these bacteria. Organisms possess a diverse range of defense mechanisms for sensing, avoiding, and removing oxidants, and *Escherichia coli* is typically used as a model for describing oxidative stress in bacterial systems (reviewed in references 79, 188, 227, and 229). However, the oxidative stress response in *N. gonorrhoeae* differs considerably from the *E. coli* paradigm (235, 236). *N. gonorrhoeae* has evolved to be highly adapted to its environment, and as a consequence it uses a novel combination of regulators and effectors to sense and respond to oxidative stress. An understanding of the mechanism of action of these systems may provide new insights into the biochemistry of oxidative stress defenses that lie outside the *E. coli* paradigm but that may be applicable to many other pathogens.

SOURCES OF OXIDATIVE STRESS

The level and type of oxidative stress that organisms are exposed to depend on the type of bacterium and the environment it inhabits. Oxidants exhibit a broad spectrum of toxic effects in biological systems, with particular species having different reactivities with DNA, proteins, and membranes (125). *N. gonorrhoeae* is exposed to oxidants that are produced both endogenously and exogenously, although some exogenously generated oxidants have the ability to penetrate cells and cause cytoplasmic damage, depending on their membrane permeability (103).

Endogenous Oxidative Stress: the Downside of Aerobic Respiration

High levels of respiration in *N. gonorrhoeae* have long been recognized as a significant potential source of endogenous ROS (9, 133). *N. gonorrhoeae* gives a positive response in the classical oxidase test that is used to distinguish between enteric bacteria and those bacteria that possess a "mitochondrial-type" respiratory chain (27). This means that electron transfer from ubiquinol to oxygen in *N. gonorrhoeae* is catalyzed by a cytochrome bc_1 complex (ubiquinol; ferricytochrome *c* oxidoreductase) and a cytochrome oxidase (ferrocytochrome *c*; oxygen oxidoreductase) (Fig. 1). Unlike almost all other aerobic bacteria, the respiratory chain of *N. gonorrhoeae* has only a single cytochrome oxidase of the cbb_3 type (the CcoNOQP cluster, gene identification no. NG1374 to NG1371; see Table 1, footnote *a*, for the "NG" gene identification website) (176; investigation of available genome sequence [Fig. 1]). The cbb_3-type oxidases are usually characterized by a high oxygen affinity, suggesting that *N. gonorrhoeae* is adapted to microaerophilic growth conditions (186). However, it is well established from research on oxidative stress in mitochondria that the cytochrome oxidase is not a major source of ROS (240).

The major source of endogenous ROS in *E. coli* is a result of auto-oxidation of components of the aerobic respiratory chain (i.e., NADH dehydrogenase [complex I] and succinate dehydrogenase [complex II], as well as fumarate reductase) (124, 167, 168, 178), leading to the generation of 5 μM/s superoxide (124, 126) (intracellular concentration, 10^{-10} M [93]) and 5 to 14 μM/s hydrogen peroxide (208) (intracellular concentration, 10^{-7} to 10^{-6} M [91, 209, 227]). It also seems likely that in *E. coli* the ubiquinol oxidases are also a source of superoxide via reactions involving quinone intermediates. However, since *N. gonorrhoeae* lacks the ubiquinol oxidases found in *E. coli*, eukaryotic mitochondria (99, 149, 164, 241, 259) represent a better comparison for the purpose of identifying sources of oxidative stress arising from quinones. Recent observations have indicated that, in addition to complexes I and II, the cytochrome bc_1 complex (complex III) of the mitochondrial

respiratory chain is a major source of superoxide (99, 149, 164, 241, 259). Since the superoxide is produced as a consequence of the reaction of ubisemiquinol with oxygen at the Q_o site of the enzyme, this means that there is considerable deposition of superoxide into the mitochondrial intermembrane space (104), equivalent to the periplasmic space in a gram-negative bacterium (125). Based on these studies of *E. coli* and eukaryotic mitochondria, NADH dehydrogenase (Nuo), succinate dehydrogenase, and the cytochrome bc_1 complex of the respiratory chain of *N. gonorrhoeae* are likely to be the main sites of endogenous ROS generation (Fig. 1).

Sera from patients with gonorrheal infection recognize both the aerobically induced protein (designated Pox 1) and an anaerobically induced nitrite reductase (AniA), indicating that *N. gonorrhoeae* is capable of both aerobic and anaerobic respiration in vivo (55, 56, 148). *N. gonorrhoeae* uses nitrite (present in cervical fluid at concentrations averaging 28 μM [242]) to support anaerobic growth (55, 143), converting nitrite (NO_2^-) to nitrous oxide (N_2O) in two reactions catalyzed by AniA (153) and nitric oxide reductase (NorB) (6, 120). The nitric oxide produced as a free intermediate during denitrification reactions is a toxic radical species (267). Nitrous oxide has been shown to be the end product of nitrite reduction (153). The genes required for formation of nitrous oxide reductase are present in the gonococcal genome (*nosLYFDZR*, NG1397 to NG1402). However, nonsense mutations in *nosZ*, *nosR*, and *nosD* indicate that an active nitrous oxide reductase is not formed in this bacterium (41).

AniA is a copper-containing nitrite reductase and is the major anaerobically induced outer membrane protein of *N. gonorrhoeae* (26, 55, 115, 165). An *aniA* null mutant is unable to grow anaerobically (119, 165). NorB of *N. gonorrhoeae* is unusual, as nitric oxide reductases are typically two-subunit enzymes encoded by *norBC*. However, as in *Ralstonia eutrophus* (62), no *norC* is seen in pathogenic *Neisseria* species (6, 120). A *norB* mutant cannot grow but can survive anaerobically, suggesting that nitric oxide is not as toxic to *N. gonorrhoeae* as it is to other organisms (120), perhaps as a consequence of redundant systems for defense against RNS (see below).

The toxic nature of nitric oxide requires that the genes involved in its production and removal be tightly regulated and induced only at low oxygen tensions to reduce the coincident generation of nitric oxide and superoxide, which can interact to generate other RNS, such as peroxynitrite (249, 267). In *E. coli*, the transcription factor FNR and the two-component regulatory systems NarQ-NarP and NarX-NarL mediate regulation of nitrate- and nitrite-induced genes (63, 193). Homologues of the *E. coli fnr*, *narQ*, and *narP* genes in *N. gonorrhoeae* have been identified (119, 153). In *N. gonorrhoeae*, AniA synthesis is regulated at the transcriptional level by FNR (114) and is upregulated by NarQ-NarP when nitrite is available (119, 153). NorB is induced by nitrite and nitric oxide but is not regulated by NarP, AniA, or anaerobiosis (120).

Recent in vitro studies, using either nitrite or a nitric oxide donor, have shown that *N. gonorrhoeae* rapidly establishes a nitric oxide steady-state level during anaerobic respiration (41). This organism is able to reduce nitric oxide levels in the surrounding medium from >1 μM (cytotoxic and proinflammatory concentration) to approximately 100 nM (anti-inflammatory concentration) (41).

Interactions of *N. gonorrhoeae* with Host Cells

N. gonorrhoeae infection is usually characterized by a symptomatic localized inflammatory response of the urethra in men (urethritis) (7, 58) and the endocervix in women (endocervicitis) (75). The purulent exudate typical of gonorrhoea contains predominantly activated polymorphonuclear neutrophils (PMNs) (7, 9). The PMN-mediated inflammatory response involves migration of PMNs toward sites of infection, phagocytosis of microorganisms, and elimination of these organisms by oxygen-dependent and oxygen-independent mechanisms (37; reviewed in references 103 and 155). Activation of PMNs results in a rapid increase in oxygen consumption, referred to as the "oxidative burst," which leads to generation of superoxide (16). This species rapidly dismutates to hydrogen peroxide and molecular oxygen (158), and the former is then consumed by myeloperoxidase, generating hypochlorous acid (252). Nitric oxide is also produced by constitutive and inducible nitric oxide synthases of PMNs (44, 146, 160), although the importance of NOS to human PMN killing remains a controversial topic. Various secondary oxidants are generated from these reactive species, including chloramines, hydroxyl radicals, singlet oxygen, and peroxynitrite (44, 103). *N. gonorrhoeae* stimulates the PMN oxidative burst (22, 177, 219, 246). However, interactions between *N. gonorrhoeae* and PMNs have not yet been fully characterized: some studies have reported that *N. gonorrhoeae* is able to survive and replicate within PMNs, while others have reported that *N. gonorrhoeae* is rapidly killed within PMNs (reviewed in references 199, 218, and 219).

In a survival assay using adherent human PMNs, a significant proportion of phagocytosed *N. gonorrhoeae* cells survived PMN killing and replicated over time, in contrast to efficient killing of *E. coli* (219). Investigation of a set of *N. gonorrhoeae* mutant strains, deficient in various oxidative stress defense mechanisms (including superoxide dismutase [SOD] and catalase) and regulatory systems, revealed that none of the oxidative defense enzymes were required on their own for *N. gonorrhoeae* to survive within PMNs in this assay (212). Another study showed that mutant strains lacking the H_2O_2-upregulated genes *recN* (involved in repair of damaged DNA; see below) and NG1686 (a putative zinc metalloprotease) had increased susceptibility to PMN killing relative to the wild type (226). These findings suggest that *N. gonorrhoeae*, like several species of bacteria, may divert the oxidative burst of PMNs (reviewed in reference 3). *Helicobacter pylori* is able to evade PMN killing despite the activation of an oxidative response by targeting of the PMN NADPH oxidase so that it locates to the plasma membrane rather than the phagosomal membrane, thus releasing ROS into the extracellular environment (4). Alternatively, oxygen-independent antimicrobial mechanisms may be of greater significance than oxygen-dependent mechanisms during PMN killing of *N. gonorrhoeae*. In support of the significance of nonoxidative killing, it has been shown that PMNs from people with chronic granulomatous disorder (NADPH oxidase deficient) had phagocytic killing capacities identical to PMNs from healthy donors (198) and that anaerobic PMNs kill as effectively as aerobic PMNs (46). *N. gonor-*

rhoeae cells exposed to serum- or phagocyte-derived lactate have increased metabolism, including increased lactate dehydrogenase activity, and rapidly consume available molecular oxygen, reducing the ability of PMNs to mount an oxidative response (29–31, 82). *N. gonorrhoeae* cells are also susceptible to the oxygen-independent components of PMN granules, including cathepsin G (45, 197, 214–217; reviewed in reference 218).

The presence of a wide range of oxidative stress defenses in *N. gonorrhoeae* may indicate that this organism encounters significant oxidative stress from sources in vivo, in addition to PMNs. The primary sites of gonococcal infection are the ecto- and endocervical epithelia in women (75) and the urethral epithelium in men (7, 58). *N. gonorrhoeae* is able to survive and replicate within epithelial cells at these sites of infection (reviewed in reference 166). Intestinal and airway epithelial cells are able to kill bacteria by oxidative mechanisms (20, 64, 200, 207). There is indirect evidence that cervical epithelial cells may also have an oxidative defense capacity, since it has been observed that several mutant *N. gonorrhoeae* strains that are susceptible to ROS killing in vitro also have decreased survival within primary human cervical epithelial cells. Such mutants include those lacking MntC (a component of the MntABC Mn uptake system) and the oxidative stress response regulator PerR (256), as well as glutathione reductase (Gor) and its regulator OxyR (K. L. Seib, H. J. Wu, Y. N. Srikhanta, J. L. Edwards, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan, and M. P. Jennings, submitted for publication).

The Host Microenvironment Inhabited by *N. gonorrhoeae*

Several commensal organisms, some of which are a significant source of ROS, coinhabit the genitourinary tract. *Lactobacillus* species, which are part of the normal vaginal flora of most women (77, 113, 163, 195), are believed to control the microflora by production of hydrogen peroxide, bacteriocins, organic acids (e.g., L-lactate) (14, 113), and nitric oxide (173, 257). Hydrogen peroxide-producing *Lactobacillus* species inhibit *N. gonorrhoeae* growth and decrease catalase activity (224, 261). Women with inhibitory strains of lactobacilli are less likely to be infected with *N. gonorrhoeae* (122, 205). Metabolism of L-lactate (produced by lactobacilli) by *N. gonorrhoeae* also greatly enhances oxygen consumption (31), and this in turn may increase levels of endogenous ROS.

DEFENSES AGAINST OXIDATIVE STRESS IN *N. GONORRHOEAE*

Defenses against oxidative stress involve constitutive and tightly regulated adaptive mechanisms to avoid and scavenge oxidants as well as to repair damaged biomolecules. Bacterial responses to oxidative stress are well defined for *E. coli* (reviewed in references 79, 188, 227, and 229). Based on this enteric model system, the generally accepted and simplified paradigm of oxidative stress defenses is that superoxide is removed by SODs (SodA, SodB, SodC), generating hydrogen peroxide in the process, which is removed by catalases (KatE, KatG) and peroxidases (AhpC). However, more than 100 proteins in *E. coli* that are involved in oxidative stress defense have been identified (95, 187). Many of these defenses are controlled by regulators that respond to iron (e.g., Fur), oxygen tension (e.g., FNR and ArcBA), superoxide (e.g., SoxRS), and hydrogen peroxide (e.g., OxyR). The complexity of the regulation of the oxidative stress response in *E. coli* is highlighted by the expression of *sodA*, which is controlled by at least six global regulators (61), and *sodB*, which is controlled by at least three regulators (72). Exposure of *E. coli* to low concentrations of hydrogen peroxide or superoxide induces a protective response that confers resistance to subsequent exposure (79, 127). Similarly, *N. gonorrhoeae* was more tolerant to oxygen when grown previously in hyperbaric pO_2 (9) or when exposed to low sublethal concentrations of ROS (82), indicating the presence of adaptive responses to ROS. The transcriptional response of *N. gonorrhoeae* to hydrogen peroxide has recently been defined; more than 150 genes are differentially regulated after transient exposure to hydrogen peroxide, and 75 of these are upregulated (226).

While *E. coli* serves as a useful model for the oxidative stress response, there are significant differences in the response of *N. gonorrhoeae* to oxidative stress. As a host-adapted pathogen, the oxidative stress response of *N. gonorrhoeae* may represent a more useful model than *E. coli* to help understand the oxidative stress responses of other human pathogens. The current state of knowledge regarding the oxidative stress defenses of *N. gonorrhoeae* is shown in Table 1 and Fig. 2 and is detailed below.

Oxidative Stress Regulons in *N. gonorrhoeae*: OxyR, PerR, and Hydrogen Peroxide

Recent advances in microarray technology and the availability of pan-neisserial genome microarrays have enabled detailed studies of the oxidative stress regulons of *N. gonorrhoeae* to be undertaken. In particular, PerR (256) and OxyR (Seib et al., submitted) regulons have been characterized through the use of *N. gonorrhoeae*/*N. meningitidis* arrays (The Institute for Genomic Research [http://pfgrc.tigr.org/]), and the transcriptome response to hydrogen peroxide (226) has been characterized using pan-neisserial arrays generated by the Neisseria Array Consortium (J. K. Davies and coworkers, personal communication).

Regulation of the peroxide stress response by OxyR is an established feature of gram-negative bacteria such as *E. coli* and *Salmonella enterica* (53, 54). On the other hand, PerR typically regulates peroxide stress responses in gram-positive organisms, including *B. subtilis* (36) and *Staphylococcus aureus* (117). The presence of both PerR and OxyR in a bacterium is rare. Apart from *N. gonorrhoeae*, such a situation has been found only in *Streptomyces coelicolor*, which has OxyR and a hydrogen peroxide-sensitive Fur-like repressor, CatR (100, 101), and *Streptomyces viridosporus*, which contains three peroxide sensor regulatory gene homologues, *ahpA*, *ahpX*, and *oxyR* (194).

Hydrogen peroxide is sensed by OxyR, a member of the LysR family of DNA-binding transcriptional modulators, which is activated by the oxidation of key cysteine residues and formation of a disulfide bond (54, 263). The OxyR regulon of *N. gonorrhoeae* consists of *gor*, *prx*, and *katA* (Table 1 and Fig. 2A; described in detail below). These three genes were differ-

TABLE 1. Defenses and regulators of the oxidative stress response of *N. gonorrhoeae*

Protein name	Function	Gene identification no.[a]	Location(s)[b]	Sensitivity of mutant strain (assay compound[s])[c]	Regulation (details of interest)[d]	Reference(s)
Oxidative stress defenses						
SodB	Superoxide dismutase B	NG0405	C	*x* (PQ, X/XO)	+ (Fur/high Fe, H_2O_2)	9, 226, 236
KatA	Catalase	NG1767	C	*s* (H_2O_2)	+ (OxyR, H_2O_2)	107, 226, 262
MntABC/Mn	ABC-type Mn transporter	NG0168	P, IM	*s* (PQ, H_2O_2)	− (PerR, Mn)	211, 236, 256
Ccp	Cytochrome *c* peroxidase	NG1769	P	*s* (H_2O_2)	+ (FNR/low O_2 + OxyR, H_2O_2)	213, 239[e]
MsrA/B	Methionine sulfoxide reductase	NG2059	C, OM	*s* (H_2O_2, X/XO)	+ (H_2O_2, Ecf σ factor)	221, 226[f]
BfrAB	Bacterioferritin	NG0794, NG0795	C	*s* (H_2O_2, PQ)	NA	49
Sco	Putative thiol:disulfide oxidoreductase/peroxiredoxin	NG1237	ND	*s* (PQ)	NA	211
GSH	Glutathione (GSH synthetase/ glutamate-cysteine ligase)	NG1217, NG0680	C	NA	NA	9
Gor	Glutathione reductase	NG0925	C	*x* (H_2O_2)	+ (OxyR, H_2O_2)	226[e]
Prx	Peroxiredoxin	NG0926	C	*xx* (H_2O_2)	+ (OxyR)	Seib et al., submitted
Laz	Azurin	NG0944	OM	*s* (H_2O_2)	NA	255
PriA	DNA replication restart helicase	NG1437	C	*s* (H_2O_2, CH)	NA	142
RecN	DNA repair	NG0318	C	*s* (H_2O_2)	+ (H_2O_2)	226
NG1686	Putative zinc metalloprotease	NG1686	ND	*s* (H_2O_2, CH)	+ (H_2O_2)	226
NG0554	*N. gonorrhoeae*-specific predicted protein	NG0554	ND	*s* (H_2O_2)	+ (H_2O_2)	226
Oxidative stress regulators						
OxyR	H_2O_2-dependent regulator	NG1813	C	*xx* (X/XO, H_2O_2)	KatA, Prx, Gor	235[e]
PerR	Mn-dependent repressor	NG0542	C	*xx* (H_2O_2)	MntABC, RpmEJ, AdhA	256
Fur	Ferric uptake regulation protein	NG1779	C	NA	Fur, SodB, BfrAB	23, 210
FNR	Fumarate/nitrate reductase regulator	NG1579	C	NA	Ccp	153, 239
NmlR	*Neisseria* merR-like regulator	NG0602	C	*s* (CH, diamide)	AdhC, TrxB, CopA	34

[a] Annotation number from the *N. gonorrhoeae* FA 1090 genome on the Los Alamos National Laboratory website (http://www.stdgen.lanl.gov/stdgen/bacteria/ngon/index.html), from the Gonococcal Genome Sequencing Project of the University of Oklahoma.

[b] C, cytoplasm; P, periplasm; IM, inner membrane; OM, outer membrane; ND, not determined.

[c] Sensitivity of mutant strains to in vitro oxidative stress killing assays relative to wild-type *N. gonorrhoeae*: *x*, same phenotype as the wild type; *s*, sensitive to killing; *xx*, resistant to killing (increased survival relative to the wild type). These assays typically involved exposure of a suspension of 10^4 to 10^6 cells over 1 h to either paraquat (PQ) (10 mM), xanthine (4.3 mM)/xanthine oxidase (300 mU/ml) (X/XO), or hydrogen peroxide (H_2O_2) (10 or 40 mM). Exposure to cumene hydroperoxide (CH) (0 to 1%) and diamide (0 to 50 mM) was performed with liquid cultures over 16 h. For further details, see the references cited in the table. NA, not available. PQ (PQ^{2+}) (1,1′-4,4′-bipyridinium dichloride) is a redox compound that is reduced to the paraquat free radical ($PQ^{\cdot+}$) by low-potential electron donors within the bacterial cell. The paraquat free radical is then oxidized by dioxygen, leading to generation of the superoxide anion ($O_2^{\cdot-}$). This redox cycling also depletes low-potential reducing agents within the cell, such as NADH (106).

[d] Regulation of defenses, either activated/upregulated (+) or repressed (−). Genes of interest, under the control of regulators, are also shown. For details, see the cited references. NA, not available.

[e] Also K. L. Seib, H. J. Wu, Y. N. Srikhanta, J. L. Edwards, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan, and M. P. Jennings, submitted for publication.

[f] Also J. K. Davies, personal communication.

entially regulated greater than twofold between the wild type and the *oxyR* mutant strain; *prx* and *gor* were downregulated, and *katA* was upregulated in the *oxyR* mutant strain relative to the wild type. Expression of these genes increases under hydrogen peroxide stress, with OxyR acting as an activator of *gor* and *prx* under high hydrogen peroxide conditions but as a repressor of *katA* under low hydrogen peroxide conditions (Seib et al., submitted). This OxyR regulon is relatively small compared to those of *E. coli* and *S. enterica* serovar Typhimurium, in which OxyR regulates expression of at least nine genes (172, 189, 266) (265). The *E. coli* regulon includes *katG* (hydroperoxidase I), *gorA*, and the peroxiredoxin *ahpCF* (alkylhydroperoxide reductase).

PerR is a member of the Fur (for "ferric iron uptake regulator") family of metalloregulatory proteins (169), and the PerR regulon typically includes antioxidant enzymes such as KatA, AhpCF, and the ferritin-like Dps protein MrgA (110). The PerR regulon of *N. gonorrhoeae* consists of 12 genes that were differentially regulated greater than twofold between the wild type and the *perR* mutant strain (Table 1, Fig. 2A). Eleven genes were upregulated in the *perR* mutant strain relative to the wild type, including the *mntABC* operon, responsible for

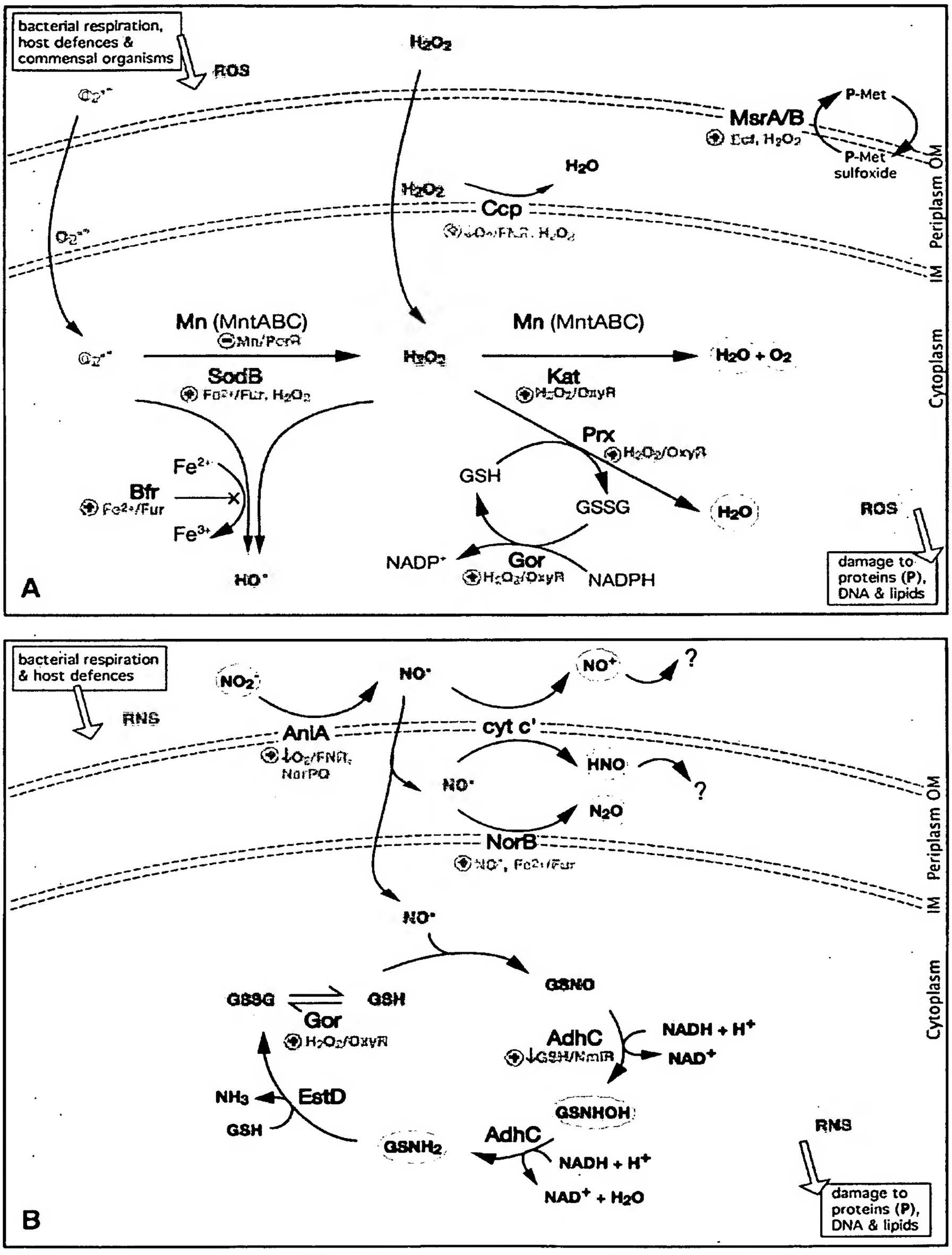

FIG. 2. Oxidative stress responses of *N. gonorrhoeae* to ROS (A) and RNS (B). The following proteins involved in oxidative stress defenses, along with their activities, are shown: MntABC, manganese transporter; Mn, manganese; SodB, superoxide dismutase B; Bfr, bacterioferritin; Kat, catalase; Ccp, cytochrome *c* peroxidase; Prx, peroxiredoxin; Gor, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MsrA/B; methionine sulfoxide reductase; NorB, nitric oxide reductase; cyt c′, cytochrome *c′*; AdhC, glutathione-dependent formaldehyde dehydrogenase; EstD, esterase D. Regulators of these defenses are shown in gray and include FNR, PerR, OxyR, Fur, NmlR, and Ecf. ROS and RNS shown include the following: $O_2^{\cdot-}$, superoxide; H_2O_2, hydrogen peroxide; $OH^{\cdot}$, hydroxyl radical; $NO^{\cdot}$, nitric oxide; GSNO, *S*-nitrosoglutathione. Products of the removal of these ROS and RNS include the following: H_2O, water; O_2, oxygen; N_2O, nitrous oxide; NH_3, ammonia. P-Met, protein methionines; +, activation; −, repression; ?, area of uncertainty with respect to certain chemical pathways.

Mn transport (see below); two genes encoding the ribosomal proteins RpmE (L31; NG0930) and RpmJ (L36; NG0931), which have been suggested to have an as-yet-unknown extraribosomal function (157); and two members of the TonB-dependent family (Tdf) of receptors, NG1205 and NG0925 (*tdfH*), which encodes a surface-exposed outer membrane protein with principal homology to heme/hemophore receptors (237). Expression of AdhA (NG1442) is reduced in the *perR* mutant, indicating that PerR acts as an activator of this gene. AdhA has considerable similarity with proteins that belong to the alcohol dehydrogenase subgroup, which contains the NAD(P)- and zinc-dependent alcohol dehydrogenases. Although known regulators of the peroxide defense response are present in *N. gonorrhoeae*, there is no evidence for the presence of SoxR in this bacterium. Thus, the pattern of gene expression in response to superoxide differs considerably from the *E. coli* paradigm.

The transcriptional response to hydrogen peroxide in *N. gonorrhoeae* involves upregulation of 75 genes and downregulation of a further 80 genes (226). Several of the genes that were upregulated encode proteins with a known role in oxidative stress defenses, including catalase, Gor and Prx (OxyR regulon), SodB (Fur regulon; see below), and MsrA/B (sigma factor Ecf regulon; see below). In addition, numerous genes involved in heat shock responses, iron acquisition (including *fur*), and regulation of transcription were shown to be upregulated (226). This study also led to the identification of several new oxidative stress defenses, including RecN (described below), NG1686 (a putative zinc metalloprotease), and NG0554 (an *N. gonorrhoeae*-specific protein of unknown function), all of which are involved in defense against peroxides.

SOD

SOD catalyzes the disproportionation of superoxide to hydrogen peroxide and water (reaction 1) (81, 161):

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

Three main classes of SOD exist, SodA (a cytoplasmic Mn-SOD), SodB (a cytoplasmic Fe-SOD), and SodC (a periplasmic Cu/Zn-SOD), all of which are found in *E. coli* (128). *E. coli* mutants lacking SOD have defects in catabolism, biosynthesis, and DNA replication (42, 84, 85).

In early studies it was observed that 80 to 100% of *N. gonorrhoeae* strains had no measurable SOD activity, and the remainder had very low SOD activity (9, 181). This appeared to contradict the accepted view that SOD was essential for all aerobic bacteria. Genes encoding SodA or SodC are not present in *N. gonorrhoeae*, but the gene encoding the complete Fe-dependent SodB (*sodB*) is present in the gonococcal genome (Table 1, Fig. 2A) (236). However, SodB does not appear to be an essential oxidative stress defense of *N. gonorrhoeae* in vitro; a *sodB* mutant showed susceptibility to superoxide killing similar to that of wild-type cells (236). *sodB* expression appears to be increased under iron-replete conditions via positive regulation by Fur (210) (described below), as seen for the similar *sodB* of *N. meningitidis* (97) and *E. coli* (73). Expression of *sodB* is also upregulated after exposure to hydrogen peroxide (226). Thus, the failure to observe superoxide dismutase activity in early investigations may have been due to iron limitation and/or a lack of induction by oxidative stress. The low activity of the Fe-SodB in *N. gonorrhoeae* suggests that the bacterium may be adapted to grow under conditions of iron limitation as a way of minimizing the production of hydroxyl radicals via Fenton chemistry (reaction 2), which would occur if ferrous iron was in contact with hydrogen peroxide:

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^- + Fe^{3+} \quad (2)$$

SodB in *E. coli* has a limited role in protection against oxidative stress, which is restricted to the transition from anaerobic to aerobic conditions (135); under aerobic conditions, SodA is of key importance. This type of regulation may be of relevance to *N. gonorrhoeae*, since this bacterium has been proposed to occupy microaerophilic or anaerobic niches (143, 153).

In contrast to *N. gonorrhoeae*, the closely related organism *N. meningitidis* contains active SodB and SodC enzymes (9, 144, 181, 213, 251). Unlike *N. gonorrhoeae*, SodB of *N. meningitidis* has been demonstrated to play a role in protection against oxidative stress (213). The predicted SodB protein sequences of *N. gonorrhoeae* and *N. meningitidis* are 96% identical. The differences seen between *sodB* mutant strains in these two species (213, 236) may be due to distinctive regulatory mechanisms in these organisms. The *sodC* gene of *N. meningitidis* is absent in *N. gonorrhoeae* and appears to have been acquired by *N. meningitidis* via horizontal transfer, most likely from commensal *Haemophilus* species that coinhabit the upper respiratory tract (144, 145). SodC of *N. meningitidis* has been reported to be involved in virulence in a mouse model (251). However, contradictory results have been reported regarding its in vitro role in defense against oxidative stress (213, 251).

Manganese and the MntABC Mn Transporter

N. gonorrhoeae uses the manganous ion (Mn^{2+}) as a chemical quenching agent of ROS in a way similar to the already-established process in *Lactobacillus plantarum*. Reactions 3 and 4 show examples of Mn quenching of ROS (Table 1, Fig. 2A) (11):

$$\begin{gathered} O_2^{\cdot -} + 2H^+ + Mn(II) \rightarrow H_2O_2 + Mn(III) \\ O_2^{\cdot -} + Mn(III) \rightarrow O_2 + Mn(II) \\ \hline 2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \end{gathered} \quad (3)$$

$$\begin{gathered} 2H_2O_2 + 2Mn(III) \rightarrow O_2 + 2H^+ + 2Mn(II) \\ 2^{\cdot}OH + 2Mn(II) \rightarrow 2OH^- + 2Mn(III) \\ \hline 2H_2O_2 + 2^{\cdot}OH \rightarrow 2H_2O_2 + O_2^{\cdot} \end{gathered} \quad (4)$$

Lactic acid bacteria lack SOD enzymes but use manganese (Mn) accumulated to millimolar concentrations intracellularly to chemically scavenge superoxide (8, 10–13). In light of the low SOD activity seen in *N. gonorrhoeae* (see above), Mn accumulation is particularly significant. This defense mechanism has also been observed in *Bacillus subtilis* mutants lacking SodA (129). Mn(II) and Mn(III) have been shown to nonenzymatically scavenge superoxide (13) and hydrogen peroxide (13, 223). The rate constant of the interaction of Mn(II) with

the peroxyl radical has been shown to be high enough to provide an antioxidant mechanism (57). In addition, the biologically relevant Mn(II)-pyrophosphate and Mn(II)-polyphosphate complexes can be effective antioxidants by indirectly decreasing or blocking hydroxyl radical production via Fenton, Haber-Weiss, xanthine oxidase-Fe-EDTA, or Fe(III)-H_2O_2-type reactions (51).

Growth of *N. gonorrhoeae* on media supplemented with Mn(II) confers resistance to in vitro oxidative killing by superoxide (236) and hydrogen peroxide (213). This phenomenon is independent of SodB and catalase, respectively, but it is dependent upon an ABC cassette-type Mn uptake system (MntABC) that is involved in this Mn accumulation and protection against ROS (236).

The MntABC Mn transporter of *N. gonorrhoeae* (Table 1, Fig. 2A) belongs to a group of recently characterized ABC permeases that are identified by the nature of the solute that is bound by their extracytoplasmic binding protein component (70). ABC transporters typically consist of three components: a periplasmic (or lipoprotein in the case of gram-positive bacteria) substrate-binding protein (MntC), two integral membrane permeases (MntB), and two peripheral membrane proteins that bind and hydrolyze ATP (MntA) (112). An *mntC* mutant of *N. gonorrhoeae* was shown to have lowered accumulation of Mn and was highly susceptible to superoxide (236)- and hydrogen peroxide (256)-induced oxidative killing compared to the wild type, even in the presence of added Mn. The *mntC* mutant grew at a reduced rate and entered stationary phase later than the wild-type strain, while an *mntAB* mutant strain had a severely defective growth rate, even when grown with Mn-supplemented media (256).

Regulation of *mntABC* expression by Mn and PerR. MntC expression is regulated by PerR (see above) and Mn in *N. gonorrhoeae* (Table 1, Fig. 2A) (256). PerR is a manganese-dependent repressor that regulates the peroxide defense response in gram-positive bacteria such as *Bacillus subtilis* (36, 111) and *Staphylococcus aureus* (117). As mentioned above, although the *perR* gene is largely restricted to gram-positive bacteria, it has been identified in *N. gonorrhoeae* (256) and *Campylobacter jejuni* (243).

An *N. gonorrhoeae perR* mutant strain is more resistant to hydrogen peroxide than is the wild-type strain, and this resistance is enhanced by supplementation of growth media with Mn(II) (256). This is similar to the phenotype of a *B. subtilis perR* mutant strain (36). In the *B. subtilis perR* mutant, catalase, alkyl hydroperoxide reductase, and the DNA binding protein MrgA are all overproduced (50). In *N. gonorrhoeae*, PerR does not appear to have a significant regulatory role in the expression of catalase (256), which is regulated by OxyR in this organism (235) (described below). However, PerR does appear to control Mn accumulation; the *perR* mutant strain has resistance to oxidative killing similar to that of the wild-type strain grown on Mn-supplemented media. Northern and Western analyses indicated that the *N. gonorrhoeae perR* mutant had higher levels of expression of the *mntC* transcript and the MntC protein than did the wild-type strain. A second transcript specific to *mntAB* was also upregulated in the *perR* mutant strain. MntC expression also appears to be repressed by Mn (256).

The regulation of *N. gonorrhoeae* MntABC is distinct from that seen in other bacteria that have been investigated, in which MntABC is regulated by a DtxR-related transcription factor, MntR. In *B. subtilis*, MntR is a Mn-dependent repressor of expression of *mntABC* and other genes involved in Mn uptake (98). MntR is divergently transcribed from *mntABC* loci in *B. subtilis* and a number of other bacteria (36, 118, 192). In *Salmonella enterica* serovar Typhimurium, expression of *sitABC* (an *mntABC* homologue) is repressed by Mn via an MntR homologue but is not responsive to oxidative stress (137). No evidence for the presence of the regulatory gene *mntR* in *N. gonorrhoeae* has been found (256). It has been suggested that the sensitivity of *N. gonorrhoeae* to Mn(II) (236) may be related to the absence of tight control of the MntABC transporter by an MntR homologue (256).

Catalase

Catalases, widespread in aerobic bacteria, are heme-cofactored enzymes that convert hydrogen peroxide to oxygen and water (reaction 5) (162, 208, 209):

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (5)$$

N. gonorrhoeae possesses very high constitutive levels of catalase (Table 1, Fig. 2A), nearly 100 times higher than *N. meningitidis* (9) and *E. coli* (107). *N. gonorrhoeae* contains a single catalase, encoded by the *katA* gene, that is located primarily in the cytoplasm, with a small concentration potentially located in the cytoplasmic membrane (107, 262). It has been demonstrated that the presence of catalase significantly increases the ability of *N. gonorrhoeae* to resist in vitro killing and DNA damage by exposure to hydrogen peroxide, human PMNs, and *L. acidophilus* (131, 132, 261, 262). A *katA* mutant strain is also significantly more sensitive to hydrogen peroxide and paraquat than is the wild type (213, 222).

Regulation of Catalase: Hydrogen Peroxide and OxyR

The catalase activity of *N. gonorrhoeae* is inducible, increasing two- to threefold when exposed to PMNs or 1 mM hydrogen peroxide (262) and 1.5-fold when exposed to hydrogen peroxide-producing *L. acidophilus* (261). However, this activity is considered weakly inducible (131). Recent analysis of the transcriptome response of *N. gonorrhoeae* to hydrogen peroxide indicated a 51.6-fold increase in levels of *katA* mRNA in cells exposed to 5 mM hydrogen peroxide (226). Expression of catalase is activated by OxyR in *E. coli*, *S. enterica* serovar Typhimurium, and other gram-negative bacteria (53, 172, 189).

A typical OxyR binding site preceding the *katA* gene of *N. gonorrhoeae* has not been found (132, 235). However, it was found that catalase expression is repressed by OxyR (see above) and is induced by hydrogen peroxide via OxyR derepression (Table 1, Fig. 2) (235). An *oxyR* mutant strain has ninefold-higher catalase activity than constitutive levels and fourfold-higher activity than the maximally induced wild-type levels and is significantly more resistant to hydrogen peroxide killing than is the wild type (235). This is distinct from the situation in *E. coli* and *S. enterica* serovar Typhimurium, in which OxyR is a positive regulator of hydrogen peroxide-inducible genes and in which increased sensitivity to hydrogen peroxide is seen in *oxyR* mutants (53, 54). Analysis of

OxyR from *N. gonorrhoeae* indicates that it contains all of the typical features of OxyR proteins. In addition, *N. gonorrhoeae* OxyR can complement an *E. coli oxyR* mutant and behave as an activator (235).

Peroxidase: Cytochrome *c* Peroxidase

Peroxidases, like catalases, are heme-cofactored enzymes responsible for hydrogen peroxide removal (208, 209). Peroxidases oxidize a reductant to convert hydrogen peroxide to water (reaction 6):

$$H_2O_2 + xH_2 \rightarrow 2H_2O + x \quad (6)$$

where x is the reductant.

N. gonorrhoeae has high peroxidase activity, some of which may be associated with the catalase protein (9, 131). However, despite similar catalase concentrations in several *N. gonorrhoeae* strains, different sensitivities to hydrogen peroxide have been seen, indicating additional mechanisms of hydrogen peroxide removal (2).

A periplasmic cytochrome *c* peroxidase (Ccp) in *N. gonorrhoeae* (Table 1, Fig. 2A) (153) that is involved in defense against hydrogen peroxide-induced killing has been reported (213, 239). The *ccp* gene of *N. gonorrhoeae* is located downstream of *katA* (see above), distal to a conserved hypothetical gene (213). An *N. gonorrhoeae ccp* mutant strain showed slight sensitivity to in vitro hydrogen peroxide killing relative to the wild-type strain; however, a *ccp/katA* double mutant strain was significantly more sensitive to in vitro hydrogen peroxide killing than was a *katA* mutant strain (213, 239). Ccp belongs to class I of the peroxidase superfamily, along with catalase-peroxidases and ascorbate peroxidases (260). It is a diheme *c*-type cytochrome that catalyzes the reduction of hydrogen peroxide using *c*-type cytochromes of the respiratory chain as the electron donor (reaction 7):

$$H_2O_2 + 2(\text{cytochrome } c_{\text{reduced}}) + 2H^+ \rightarrow 2H_2O + 2(\text{cytochrome } c_{\text{oxidized}}) \quad (7)$$

In *E. coli*, molecular oxygen is sensed by the transcription factor FNR (21). FNR is essential for the gonococcal response to oxygen (153). Ccp of *N. gonorrhoeae* is expressed under conditions of low oxygen tension/anaerobiosis (153) and is dependent on FNR (239). Expression of *ccp* is also upregulated by hydrogen peroxide (226) and PerR (256).

Ccp of *N. gonorrhoeae* is believed to be a lipoprotein, with a signal peptide for cleavage by signal peptidase II, that is anchored to the membrane in the periplasm (239). The lipid modification has been suggested as a way of maintaining the close proximity of Ccp to its *c*-type cytochrome electron donors located in, or associated with, the cytoplasmic membrane, while also maintaining it in a location where its protective mechanism is required (239). However, *N. gonorrhoeae* Ccp may also be involved in energy generation by using hydrogen peroxide as an electron acceptor, as in the yeast *Hansenula polymorpha* (244).

In methylotrophic yeast, Ccp can substitute for catalase. Increased Ccp levels can suppress the phenotype of a catalase-deficient mutant strain (90). In addition, the presence of abundant Ccp activity has been suggested to compensate for the natural absence of catalase in *Fasciola hepatica* and *Schistosoma mansoni*. Partially purified Ccp from these organisms can inhibit damage induced by oxidative stress in vitro (38). Ccp has been identified in several bacteria, including *Pseudomonas aeruginosa* (83), *Pseudomonas stutzeri*, *Paracoccus denitrificans* (92), and *Rhodobacter capsulatus* (121), although functional roles have not yet been determined for Ccp in these organisms. Analysis of the distribution of Ccp in *Neisseria* spp. indicated that it is widespread in *N. gonorrhoeae* and commensal *Neisseria* strains but is absent from all *N. meningitidis* strains (213).

N. gonorrhoeae does not appear to contain the peroxidases present in other bacteria. *E. coli* and *S. enterica* serovar Typhimurium possess an NADPH-dependent alkyl hydroperoxide reductase (AhpC) capable of reducing organic peroxides to alcohols (130, 228). Ahp scavenges the majority of the endogenous hydrogen peroxide in *E. coli* (208). *E. coli* also possesses a thiol peroxidase (47). *N. meningitidis* contains a constitutively expressed cytoplasmic glutathione peroxidase (Gpx) (1, 171) that is involved in oxidative defense (170). An *N. meningitidis gpx* mutant strain is highly sensitive to paraquat and slightly sensitive to hydrogen peroxide (170). However, Gpx is absent from *N. gonorrhoeae* and the commensal *Neisseria* species investigated (171).

Thiol-Based Defenses

GSH and Gor. The low-molecular-weight compound glutathione (γ-L-glutamyl-L-cysteinylglycine) (GSH) is considered one of the first lines of defense against oxidative stress (188). GSH, typically present in cells at millimolar concentrations (5 mM in *E. coli*) (191), is a chemical scavenger of radicals and acts as a hydrogen donor to restore oxidized macromolecules (43). Very high concentrations of GSH (reported to be >15 mM) are present in *N. gonorrhoeae*, which may constitute a powerful antioxidant system (9). The GSH oxidoreductase (Gor) (NG0925), which typically maintains the reduced pool of GSH (43), has recently been identified in *N. gonorrhoeae* as part of the OxyR regulon (Table 1, Fig. 2; described above). As in *E. coli* (266), Gor is upregulated by OxyR under conditions of increased hydrogen peroxide stress (Seib et al., submitted). Gor is annotated in the *N. gonorrhoeae* genome as a dihydrolipoamide dehydrogenase (DldH), due to its similarity to this family of proteins. The *dldH* gene, identified by Stohl et al. (226) to be upregulated by hydrogen peroxide, is the same gene that encodes Gor (Seib et al., submitted).

Thioredoxin and glutaredoxin. The thiol donors thioredoxin (Trx) and glutaredoxin (Grx) are small proteins with conserved cysteine pairs that are oxidized to cystine disulfides upon reduction of cellular proteins. These thiol proteins are members of the cytoplasmic thioredoxin superfamily, the best characterized members of which are thioredoxins 1 and 2 (TrxA and TrxC, respectively) and glutaredoxins 1, 2, and 3 (GrxA, GrxB, and GrxC, respectively) (15). Reduced Trx and Grx are regenerated by an NADPH-dependent thioredoxin reductase and a GSH-dependent Gor, respectively (15). *E. coli* mutants lacking these pathways grow poorly under aerobic conditions (191). Several genes in the *N. gonorrhoeae* genome sequence (156) have been annotated as thioredoxins (NG0652, *trx1*, thioredoxin I; NG1923, *tlpA*, thioredoxin; NG0057, thioredoxin-like protein; NG0331, possible thioredoxin) and glutaredoxins

(NG1381, *grx2*, glutaredoxin 2; NG0114, *grx3*, glutaredoxin 3; NG0351, probable glutaredoxin-related protein). Expression of *trx1*, *grx2*, and *grx3* is upregulated by exposure to hydrogen peroxide (226). A potential thioredoxin reductase (NG0580, *trxB*) has also been identified. Gor of *N. gonorrhoeae* is described above.

Peroxiredoxin. Peroxiredoxins (Prx) are nonheme enzymes that catalyze alkyl hydroperoxide reduction via conserved reactive cysteines, which are typically regenerated by a Trx or Grx thiol reductant (116). The cytoplasmic cofactor NADPH is essential in maintaining the reducing power of the cell for these systems, and enzymes that are involved in maintaining NADPH are upregulated by oxidative stress (187).

A hybrid peroxiredoxin/glutaredoxin (Prx) in *N. meningitidis* that is active in the reduction of various peroxides, including hydrogen peroxide and dehydroascorbate, in the presence of GSH has been isolated (202). The hybrid protein contains a Prx module in the N terminus and a Grx module in the C terminus joined by an eight-amino-acid linker peptide; however, the interaction of these domains remains unclear (202). The Prx protein of *N. gonorrhoeae* has recently been identified as part of the OxyR regulon; its expression is upregulated by hydrogen peroxide-activated OxyR (Table 1, Fig. 2A; see above) (Seib et al., submitted). A *prx* mutant strain has increased levels of catalase and is resistant to hydrogen peroxide killing (Seib et al., submitted).

Sco. A homologue of the yeast Sco (for "synthesis of cytochrome oxidase") protein in *N. gonorrhoeae* was identified and found to be a novel protein involved in oxidative stress defense (Table 1). *N. gonorrhoeae sco* mutant strains were highly sensitive to in vitro paraquat-induced oxidative killing, indicating that Sco is involved in protection against oxidative stress in these bacteria (211).

Members of the SCO1/SenC family (Pfam 02630 [19]) are considered to be involved primarily in biogenesis of the Cu_A center of aa_3-type cytochrome oxidases via an as-yet-undefined role in copper storage or transport (68, 89, 159, 180, 184, 185, 196). However, *N. gonorrhoeae* does not possess an aa_3-type cytochrome oxidase with a Cu_A center (see above), and an *sco* mutant strain is unaffected in cytochrome oxidase activity. It has been proposed that SCO1/SenC proteins have thiol:disulfide oxidoreductase or peroxiredoxin activity and play a catalytic role as an antioxidant protein in the periplasm (52). Primary- and secondary-structure predictions of *N. gonorrhoeae* Sco (211), as well as the recently determined protein structure of the *B. subtilis* Sco protein (17), indicate that these proteins are structurally related to peroxiredoxins and thiol:disulfide oxidoreductases. Thiol:disulfide oxidoreductases and peroxiredoxins are involved in protection against oxidative stress in several bacteria (48, 116, 140).

Methionine sulfoxide reductase. Cysteine and methionine, the two thiol-containing amino acids that occur in proteins, play a role in several antioxidant systems. Methionine residues are highly susceptible to oxidation by superoxide, hydrogen peroxide, and nitric oxide, resulting in formation of methionine sulfoxide residues in proteins (247). Methionine sulfoxide reductase (Msr) is capable of catalyzing the reduction of methionine sulfoxide residues back to methionine (32, 33, 150, 225). *N. gonorrhoeae* produces two forms of Msr, a cytoplasmic form and a form with a signal sequence that is secreted to the outer membrane, which is involved in protection from hydrogen peroxide and superoxide (Table 1, Fig. 2A) (221, 230, 253). Similarly, Msr of *E. coli* is involved in protection from hydrogen peroxide and nitric oxide (174, 225).

MsrA and MsrB, specific for separate methionine sulfoxide epimers, are often separate proteins; however, they are encoded as a fused protein in a single open reading frame in *N. gonorrhoeae*, with an N-subdomain disulfide oxidoreductase, a central MsrA, and a C-subdomain thioredoxin-dependent MsrB (183, 221). MsrA/B was initially designated PilB, a regulator of pilin expression (231), but this has since been disproved (221).

Methionine oxidation has been implicated in conformational changes and inactivation of proteins (247). However, methionine residues are considered endogenous antioxidants in proteins due to the ability to be reduced and regenerated by Msr (150). In *E. coli*, Msr is in turn reduced by the thioredoxin system (225); however, given the location of the gonococcal Msr, the question of how it receives its reducing power is an open one. Msr is also believed to be involved in maintenance of adhesins, which are vital for colonization and virulence, in bacterial pathogens (108, 253).

Expression of *msrA/B* is upregulated in response to hydrogen peroxide (226), and the single extracytoplasmic function sigma factor (Ecf) of *N. gonorrhoeae* has also been shown to positively regulate expression of *msrA/B* (J. K. Davies, personal communication). The way in which oxidative stress is sensed in this system is not known, but presumably it is controlled by the interaction of an anti-sigma factor and the Ecf protein, as in other systems (reviewed in reference 18).

Azurin

Azurin is a small, blue, copper-containing protein that functions in electron transport during respiration in several different microorganisms (80, 134, 203, 204). Electrons are typically passed through the quinone pool and cytochrome bc_1 and via periplasmic electron shuttles such as *c*-type cytochromes and azurin to nitrite reductase (71, 267). However, azurin of *P. aeruginosa* is not essential for denitrification but is involved in protection from oxidative stress (245). A *P. aeruginosa azu* mutant was proven to be very sensitive to ROS, including hydrogen peroxide and superoxide radicals.

An azurin homologue, *laz* (for "lipid-modified azurin"), has been identified in both *N. gonorrhoeae* (Table 1) (94) and *N. meningitidis* (254). The *P. aeruginosa* azurin is not a lipoprotein (40). Laz is tethered to the outer membrane via palmityl fatty acid (234, 254) and possesses an N-terminal domain consisting of five imperfect repeats of the sequence Ala-Ala-Glu-Ala-Pro (AAEAP), found in the H.8 protein, and a C-terminal domain very similar to that of azurin of other bacteria (94, 136). Laz is not believed to play a direct role in respiration, but may function in an as-yet-unidentified electron transport pathway in pathogenic *Neisseria* species (39). The *N. gonorrhoeae* and *N. meningitidis laz* mutants were more sensitive to hydrogen peroxide, but not superoxide, than was the wild type (255). These mutants also had increased sensitivity to copper toxicity, suggesting that Laz may play an important role in copper sequestration (255).

Iron Sequestration

Iron, although required by aerobic cells, is also problematic due to its ability to catalyze Fenton and Haber-Weiss reactions that generate ROS. Consequently, iron transport is tightly regulated and free iron is maintained at low concentrations within cells (76). In human cells, intracellular iron is sequestered mainly in ferritin while extracellular iron is bound to lactoferrin and transferrin. Bacteria produce scavenging proteins to acquire iron in iron-limited host environments. Intracellular bacterial iron is complexed primarily to ferritin or to the heme-containing bacterioferritin (179). Overloading of bacterial cells with iron leads to accelerated DNA damage (138, 233). Conversely, iron deprivation has been shown to protect *N. gonorrhoeae* from ROS (59).

N. gonorrhoeae is able to use multiple host iron-binding proteins as a direct source of iron, and the organism contains several high-affinity iron storage and transport proteins that maintain low levels of free intracellular iron (reviewed in references 86 and 201). Bacterioferritin (Bfr) of *N. gonorrhoeae*—composed of two subunits, BfrA (ferroxidase center) and BfrB (heme-binding ligand)—is involved in iron storage, growth under iron-limited conditions, and defense against iron-mediated oxidative stress generated by hydrogen peroxide and paraquat (Table 1, Fig. 2A) (49). Most bacteria possess either a ferritin or Bfr homologue (49), although *E. coli* possesses both (5). Investigation of genome databases has not revealed a ferritin homologue in *N. gonorrhoeae* (49).

Regulation of iron uptake by Fur. Microarray analysis has indicated that 203 genes are regulated in response to iron in *N. gonorrhoeae*: 109 genes are upregulated and 94 genes are downregulated by iron deprivation (74). Based on analysis of putative promoter regions, 50 operons were predicted to be directly regulated by Fur (74). The expression of the majority of iron binding proteins is regulated by Fur in *N. gonorrhoeae* (23) in a manner similar to that of several other bacteria (78). The Fur regulon of *N. gonorrhoeae* includes the iron-repressible genes *tbpB*, *lbpB*, *tonB*, and *fbpA*, which are associated with iron sequestration under iron-limiting conditions (74, 86). It has proved more difficult to identify genes under Fur control that are activated in response to high levels of iron. Attempts to generate a Fur null mutant have so far been unsuccessful, which may indicate that Fur plays a critical role in gonococcal survival (232). However, *fur* has since been successfully deleted in *N. meningitidis* (65), and attempts to disrupt Fur in *N. gonorrhoeae* may have failed due to a polar effect on an essential downstream gene. Many iron-responsive genes that have been identified in *N. gonorrhoeae* may not be directly regulated by Fur. However, analysis of *N. meningitidis* has identified at least 15 genes whose levels are increased in the presence of high iron levels and which contain a Fur box in their promoter regions (97). These genes include *sodB*, *recN* (see below), *aniA*, and *norB*. BfrAB (see above) is also potentially regulated by Fur (49).

In *E. coli*, *fur* autoregulates its expression in response to iron levels, but it is also controlled by OxyR and the SoxR/S system and is induced by oxidative stress (264). By lowering iron uptake during oxidative stress, *E. coli* reduces the potential damage that would be caused by Fenton chemistry involving a combination of Fe(II) and hydrogen peroxide. Expression of *fur* does not appear to be regulated by OxyR or PerR in *N. gonorrhoeae*, as indicated by microarray analysis (see below) (256) (Seib et al., submitted). However, *fur* and several of the genes within the Fur regulon are upregulated in response to hydrogen peroxide (226).

The *N. gonorrhoeae* transcriptome response to hydrogen peroxide, described by Stohl and coworkers (226), confirms a link between iron and hydrogen peroxide regulation; however, a more detailed knowledge of individual regulons is required before the pattern of gene expression following the addition of peroxide can be understood fully. This problem is highlighted by the observation that upon the addition of hydrogen peroxide to *N. gonorrhoeae* there was increased expression of genes that are repressed by Fur (iron acquisition genes) and those that are probably activated by Fur (*sodB*, *recN*). Assuming that Fe-Fur is the active form of this transcription factor, it is hard to see how derepression and induction of genes can occur at the same time. It is notable that the collection of data for this experiment involved RNA isolation after a 15-min exposure to hydrogen peroxide (226). As a consequence, there is the possibility that complex changes in gene expression, not to mention changes in mRNA stability, may have been missed in this single-time-point analysis. Of particular relevance here may be changes in the intracellular iron pool in response to oxidative stress. The complex pattern of gene expression in response to hydrogen peroxide highlights the need for investigation of the kinetics of changes in gene expression at a global level and for careful analysis of these regulons.

DNA Repair Mechanisms

It has been suggested that free radicals generated during aerobic respiration are a major source of DNA damage in *N. gonorrhoeae* (142). *N. gonorrhoeae* has a network of DNA repair strategies that includes base excision, nucleotide excision, and mismatch and recombinational repair mechanisms. Comparison of *Neisseria* DNA repair genes with the well-characterized DNA repair mechanisms of *E. coli* has indicated that there are no gross differences in the repair capabilities of these organisms, with the exception of the absence of the SOS response in *Neisseria* (reviewed in reference 141).

PriA, a helicase of *N. gonorrhoeae*, is predicted to deal directly with oxidative stress-induced DNA damage through its role in restarting DNA replication at stalled replication forks (Table 1) (142). A *priA* mutant strain of *N. gonorrhoeae* has decreased DNA repair capability and is sensitive to hydrogen peroxide and cumene hydroperoxide killing relative to the wild-type parental strain (142). RecN, involved in repair of damaged DNA (220), also appears to be directly involved in defense against oxidative stress in *N. gonorrhoeae* (Table 1). A *recN* mutant strain has increased sensitivity to hydrogen peroxide and PMN killing (226). RecN is positively regulated by Fur (described above) (210) and hydrogen peroxide (226).

Defenses against RNS

Bacterial responses to RNS, while not as well characterized as the responses to ROS, are increasingly recognized as being diverse and critical for bacterial survival. In many cases, RNS resistance mechanisms overlap defenses against ROS; for ex-

ample, in *E. coli*, SoxR is activated by both superoxide (96, 152) and nitric oxide (69, 182). The prominent nitric oxide-detoxifying enzymes in *E. coli* appear to be flavohemoglobin (HmpA) and the flavorubredoxin/flavorubredoxin reductase (NorVW) system, regulated by a nitric oxide response regulator, NorR (175). These systems do not seem to exist in *N. gonorrhoeae*. The periplasmic nitrite reductase (NrfA) of *E. coli* has also been shown to reduce nitric oxide (190). This enzyme is a multiheme cytochrome and is distinct from the gonococcal nitrite reductase, AniA (Fig. 2B; see above). Nitric oxide reductase, which catalyzes the reduction of nitric oxide to the less toxic compound nitrous oxide, plays a major role in protecting organisms from nitric oxide (Fig. 2B) (reviewed in reference 267). A knockout mutation of nitric oxide reductase of *P. stutzeri* is lethal, but the effect can be suppressed by a further mutation that inactivates the nitric oxide generator (267). The nitric oxide reductase (NorB) of *N. meningitidis* is able to counteract toxicity due to exogenously added nitric oxide (6). A NorB mutant of *N. gonorrhoeae* is still resistant to nitric oxide (120), indicating that there are additional detoxification pathways present in the bacterium. Cytochrome *c'* (CycP; NG108), an outer membrane lipoprotein that binds nitric oxide in vitro, is believed to protect *N. gonorrhoeae* from endogenously and/or exogenously generated nitrosative stress (Fig. 2B) (238). A *cycP* mutant strain of *N. gonorrhoeae* has an extended lag phase during microaerobic growth in the presence of nitrite. It is suggested that the constitutive synthesis of cytochrome *c'* provides an instant defense by binding nitric oxide until NorB has accumulated to levels sufficient to reduce nitric oxide and by preventing the generation of peroxynitrite from free nitric oxide and superoxide (238). Cytochrome *c'* has also been shown to bind nitric oxide and to act as a buffer against this toxic radical species in *N. meningitidis* (6) and *P. denitrificans* (249). However, cytochrome *c'* may be of limited defense against cytoplasmic nitric oxide.

AhpC is an NADPH-dependent alkyl hydroperoxide reductase, reducing an alkyl (or hydrogen) peroxide to water and alcohol (or water). In both *E. coli* and *S. enterica* serovar Typhimurium, AhpC also acts as a peroxynitrite reductase (35), but no AhpC protein is annotated in the gonococcal genome. However, the bacterioferritin comigratory protein (Bcp; NG0328) of *N. gonorrhoeae* is highly similar in sequence to AhpC of *E. coli* (27% identical and 45% similar over 104 of 167 amino acids) and may be functionally similar. The methionine sulfoxide reductase (MsrA) in the cytoplasm of *E. coli* has also been proposed to be involved in the repair of methionines damaged by peroxynitrite (225), representing an indirect defense against nitric oxide. It is notable that *N. gonorrhoeae* possesses a methionine sulfoxide reductase (MsrA/B; see above) in its outer membrane. Although the gonococcal *msrA/B* mutant was shown to be highly sensitive to oxidative stress, it has not been tested in its sensitivity to RNS (221).

Regulation of RNS defenses. OxyR and SoxRS are the established regulators of the hydrogen peroxide and superoxide responses in *E. coli* (79). Both of these regulators have been shown to also respond to RNS (66, 109). It is worth emphasizing that the mechanisms and roles of these regulators differ among bacterial systems. Indeed, *N. gonorrhoeae* does not appear to possess the SoxRS system, and it is unusual in that its OxyR protein is a repressor of catalase (235) rather than an activator, as in enteric bacteria (54). Although SoxR has been suggested to have a role in nitric oxide sensing, recent microarray experiments with *E. coli* have shown that the response to nitrosative stress is controlled primarily by NorR (the regulator of NorV/W) and Fur (175). *N. gonorrhoeae* lacks the NorR regulator and NorV/W, and this has led to a search for the systems that protect this bacterium against RNS.

Recently, a novel MerR-like transcription factor, NmlR (for "*Neisseria* MerR-like Regulator") (NG0602), which is an activator upon disulfide or redox stress and appears to be a key regulator in the defense against RNS, was identified in *N. gonorrhoeae* (Fig. 2B) (139). NmlR regulates the expression of *adhC* (NG0601), which encodes a zinc-dependent alcohol dehydrogenase (AdhC) that appears to be able to catalyze the reduction of *S*-nitrosoglutathione (GSNO) (Fig. 2A). This enzyme is present in both eukaryotes and *E. coli*, where it has demonstrated activity to remove GSNO (154), although its regulation is different. GSNO is a spontaneously formed adduct between reduced glutathione and nitric oxide; AdhC activity therefore controls the level of *S*-nitrosylated proteins and provides defense against the stresses exhibited by RNS. An *N. gonorrhoeae nmlR* mutant was more susceptible to killing by cumene hydroperoxide and diamide than were wild-type cells and had decreased growth under microaerophilic conditions (139). The *nmlR* mutant is also sensitive to killing by nitric oxide (A. J. Potter, S. P. Kidd, and A. G. McEwan, unpublished observations). The current model for the action of NmlR is that it is a Zn-dependent transcription factor which acts as a repressor of gene expression when it is in the holo form. Upon disulfide stress induced by a variety of electrophiles, such as nitric oxide, aldehydes, or peroxides, it is proposed that NmlR loses Zn and the apo form, which may contain at least one disulfide bridge, acts as an activator of gene expression. This represents a classical MerR-like response mechanism, the switch from a repressor to an activator.

NmlR also regulates a metal ion efflux pump (annotated as CopA; NG0579) and an annotated thioredoxin reductase (TrxB; NG0580) (see above), although the role of these proteins in this novel defense system is not yet defined. Analysis of microbial genomes has defined an NmlR subfamily of the MerR-like regulators, all of which are associated with *adhC*-like genes (139). MerR family proteins do not typically sense redox changes or ROS in the cell, with the exception of SoxR, which contains a superoxide-sensitive iron-sulfur cluster (189).

CONCLUDING REMARKS

Our understanding of the oxidative stress response of *N. gonorrhoeae* has greatly advanced in recent years. This understanding has continuing importance, as gonorrhea remains a serious health risk and has been linked to infertility and increased transmission and susceptibility to human immunodeficiency virus infection (60, 88, 147, 151, 248). Central to this bacterium's ability to persist and cause disease is an ability to overcome the oxidative stress generated in and around the host-pathogen environment.

It has become evident that *N. gonorrhoeae* has evolved complex, and in some cases novel, mechanisms to cope with oxidative and nitrosative stress (Table 1, Fig. 2). The primary defenses used by *N. gonorrhoeae* against ROS include accumu-

lation of manganese by the MntABC transport system (236), unusually high catalase and peroxidase activities (9, 132, 213, 239, 262), and very high concentrations of GSH (9). However, further work is required to better understand the RNS defenses of this organism. Five environmental sensors that are involved in the oxidative stress response have been described to date: the superoxide-dependent OxyR (235), the Mn-dependent PerR (256), the Zn-dependent thiol-based NmlR (139), the iron-dependent Fur (23, 86, 210), and the oxygen-dependent FNR (153, 239). Further characterization of the regulons of these environmental sensors will provide a better understanding of the coordinated response of *N. gonorrhoeae* to oxidative and nitrosative stress.

It is clear that *N. gonorrhoeae* has mechanisms to defend against ROS and/or RNS at the cell surface, in the periplasm, and in the cytoplasm (Fig. 2). *N. gonorrhoeae* has the ability to defend against nitric oxide in these three cellular locations (Fig. 2B). There are no obvious dedicated defense systems against superoxide in the outer membrane, but Sco, which faces the periplasm, may provide some protection against this form of ROS (211). It is interesting that the pK_a for the peroxyl-superoxide interconversion is 4.8; thus, under the acidic conditions of the cervix, the peroxyl radical ($HO_2\cdot$) represents about 50% of the superoxide species outside the cytoplasmic membrane. This neutral form of superoxide is much more permeable across the cytoplasmic membrane, where it forms superoxide by the loss of a proton. Adaptation to a combination of low pH and chronic oxidative stress in the female urogenital tract may be the reason the gonococcus has evolved such an unusual Mn-based defense system against superoxide. It is also interesting that the other superoxide defense system in *N. gonorrhoeae* (SodB) is regulated by iron and does not appear to have a key role in vitro. It is possible that under more iron-replete conditions, SodB would have a key role; it is tempting to speculate that this may be inside a PMN or cervical epithelial cell. Defense against peroxide is also critical, and again it appears that key defenses are associated with the cytoplasm (Fig. 2A). Finally, more work is required to identify the components that provide defense against secondary ROS and RNS products such as peroxynitrite and reactive aldehyde species.

The oxidative stress defenses of *N. gonorrhoeae* have typically been characterized based on the sensitivity of mutant strains to in vitro oxidative killing. However, recent studies using primary human cell lines have revealed interesting findings with respect to the biological significance of several of these oxidative stress responses. A set of mutant strains of *N. gonorrhoeae*, deficient in various oxidative stress defense mechanisms or regulatory systems, was not sensitive to PMN killing in an adherent PMN phagocytosis assay relative to the wild-type strain, despite the stimulation of the oxidative burst by *N. gonorrhoeae* (212). However, examination of the same panel of mutant strains in a primary human cervical epithelial cell line revealed that strains lacking PerR, MntC (256), OxyR, or Gor (Seib et al., submitted) had decreased survival. These findings raise important questions about the major species and sources of oxidative stress that *N. gonorrhoeae* is exposed to during infection. PMN-*N. gonorrhoeae* interactions have a controversial history (reviewed in references 199, 218, and 219), but it has been established that an oxidative burst is stimulated in response to infection by *N. gonorrhoeae* (22, 177, 219, 246). Therefore, it will be interesting to determine how *N. gonorrhoeae* survives PMN oxidative killing, in which several major characterized defenses in vitro do not seem to play a role in vivo. Intestinal and airway epithelial cells are able to kill bacteria by oxidative mechanisms (20, 64, 200, 207), and oxidative stress in urogenital epithelial cells may be a more significant source of oxidative stress than previously recognized. With the lack of relevant animal models, further studies using primary human cell lines may provide answers to some of the questions posed.

ADDENDUM IN PROOF

Recently, we have identified *nmlR* and *adhC* homologues in *Streptococcus pneumoniae* and *Haemophilus influenzae*. An *adhC* mutant of *Streptococcus pneumoniae* and *Haemophilus influenzae* is sensitive to killing by GSNO (S. P. Kidd, M. P. Jennings, and A. G. McEwan, unpublished observations). This contrasts with the situation for *N. gonorrhoeae*, for which an *adhC* mutant shows essentially the same sensitivity to GSNO as wild-type cells. This has led us to conclude either that AdhC is of minor importance in the defense of *N. gonorrhoeae* against NO killing or that there are redundant systems for NO defense in this bacterium. We note that Nikitovic and Holmgren (J. Biol. Chem. 271:19180–19185, 1996) have observed that GSNO can be cleaved by the reduced thioredoxin, generating NO and superoxide. It is interesting that the source of reducing power for thioredoxin, NADPH-dependent thioredoxin reductase, is encoded by a gene (*trxB*) that is also regulated by NmlR. Thus, an alternative route for dealing with GSNO may operate in *N. gonorrhoeae*.

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Article V

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REVIEW ARTICLES

Cellular Response to Oxidative Stress: Signaling for Suicide and Survival

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Reactive oxygen species (ROS), whether produced endogenously as a consequence of normal cell functions or derived from external sources, pose a constant threat to cells living in an aerobic environment as they can result in severe damage to DNA, protein, and lipids. The importance of oxidative damage to the pathogenesis of many diseases as well as to degenerative processes of aging has becoming increasingly apparent over the past few years. Cells contain a number of antioxidant defenses to minimize fluctuations in ROS, but ROS generation often exceeds the cell's antioxidant capacity, resulting in a condition termed oxidative stress. Host survival depends upon the ability of cells and tissues to adapt to or resist the stress, and repair or remove damaged molecules or cells. Numerous stress response mechanisms have evolved for these purposes, and they are rapidly activated in response to oxidative insults. Some of the pathways are preferentially linked to enhanced survival, while others are more frequently associated with cell death. Still others have been implicated in both extremes depending on the particular circumstances. In this review, we discuss the various signaling pathways known to be activated in response to oxidative stress in mammalian cells, the mechanisms leading to their activation, and their roles in influencing cell survival. These pathways constitute important avenues for therapeutic interventions aimed at limiting oxidative damage or attenuating its sequelae. J. Cell. Physiol. 192: 1–15, 2002. Published 2002 Wiley-Liss, Inc.†

For organisms living in an aerobic environment, exposure to reactive oxygen species (ROS) is continuous and unavoidable. ROS encompass a variety of partially reduced metabolites of oxygen (e.g., superoxide anions, hydrogen peroxide, and hydroxyl radicals) possessing higher reactivities than molecular oxygen (reviewed in Thannickal and Fanburg, 2000). They are generated intracellularly through a variety of processes, for example, as byproducts of normal aerobic metabolism, or as second messengers in various signal transduction pathways. They can also be derived from exogenous sources, either being taken up directly by cells from the extracellular milieu, or produced as a consequence of the cell's exposure to some environmental insult. Transient fluctuations in ROS serve important regulatory functions, but when present at high and/or sustained levels, ROS can cause severe damage to DNA, protein, and lipids. A number of defense systems have evolved to combat the accumulation of ROS. These include various non-enzymatic molecules (e.g., glutathione, vitamins A, C, and E, and flavenoids) as well as enzymatic scavengers of ROS (e.g., superoxide dismutases (SOD), catalase, and glutathione peroxide). Unfortunately, these defense mechanisms are not always adequate to counteract the production of ROS, resulting in what is termed a state of oxidative stress. Oxidative stress has been implicated in a wide variety of disease processes including atherosclerosis, diabetes, pulmonary fibrosis, neurodegenerative disorders, and arthritis, and is believed to be a major factor in aging (reviewed in Finkel and Holbrook, 2000).

At the cellular level, oxidant injury elicits a wide spectrum of responses ranging from proliferation to growth arrest, to senescence, to cell death (see Fig. 1). The particular outcome observed can vary significantly from one cell type to the next, as well as with respect to the agent examined, its dosage and/or duration of treatment. However, whatever the effect seen, it largely reflects the balance between a variety of intracellular stress signaling pathways that are activated in response to the oxidative insult. As indicated in Figure 2, these pathways exert their phenotypic influences largely through modulation of transcription factor activities that effect changes in the pattern of gene expression.

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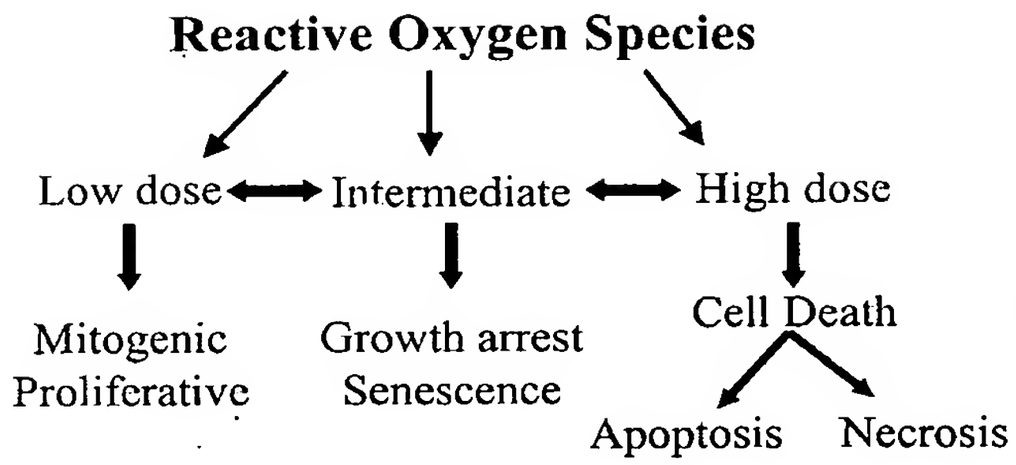

Fig. 1. Reactive oxygen species (ROS) elicit a wide spectrum of responses. These responses depend upon the severity of the damage, which is further influenced by the cell type, the magnitude of the dose, and the duration of the exposure. Typically, low doses of ROS, particularly hydrogen peroxide, are mitogenic and promote cell proliferation, while intermediate doses result in either temporary or permanent growth arrest, such as replicative senescence. Very severe oxidative stress ultimately causes cell death via either apoptotic or necrotic mechanisms. As discussed throughout this review, a large number of signaling pathways are involved in coordinating the response to elevations in ROS and influencing the particular course taken by a cell.

Some of the pathways are clearly linked to enhanced survival, while others are associated with cell death. Still others can produce either effect dependent on the circumstances. In this review, we discuss the major signaling pathways known to be involved in regulating the cellular response to oxidative stress, focusing on the specific mechanisms contributing to their activation by oxidants as well as their roles in influencing cell survival during oxidant injury.

MITOGEN-ACTIVATED PROTEIN KINASES

Mitogen-activated protein kinases (MAPKs) encompass a large number of serine/threonine kinases involved in regulating a wide array of cellular processes including proliferation, differentiation, stress adaptation, and apoptosis. Based on structural differences, they are divided into three multimember subfamilies: the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 kinases. The ERK, JNK, and p38 subfamilies are activated via independent (though sometimes overlapping) signaling cascades involving a MAPK kinase (MAPKK) that is responsible for phosphorylation of the MAPK, and a MAPK kinase kinase (MAPKKK) that phosphorylates and activates MAPKK. MAPKs mediate their effects through phosphorylation of a wide range of effector proteins, most notably transcription factors, which in turn lead to changes in the pattern of gene expression. The ERK pathway is most commonly linked to the regulation of cell proliferation, while the JNK and p38 pathways are more strongly tied to stress. For this reason, and because they are frequently activated in a coordinate fashion, JNK and p38 are often grouped together and referred to as stress-activated protein kinases (SAPK). Detailed reviews of the various pathways can be found elsewhere (Kolch, 2000; Chang and Karin, 2001; Kyriakis and Avruch, 2001).

The ERK, JNK, and p38 subfamilies have all been shown to be activated in response to oxidant injury and therefore could potentially contribute to influencing

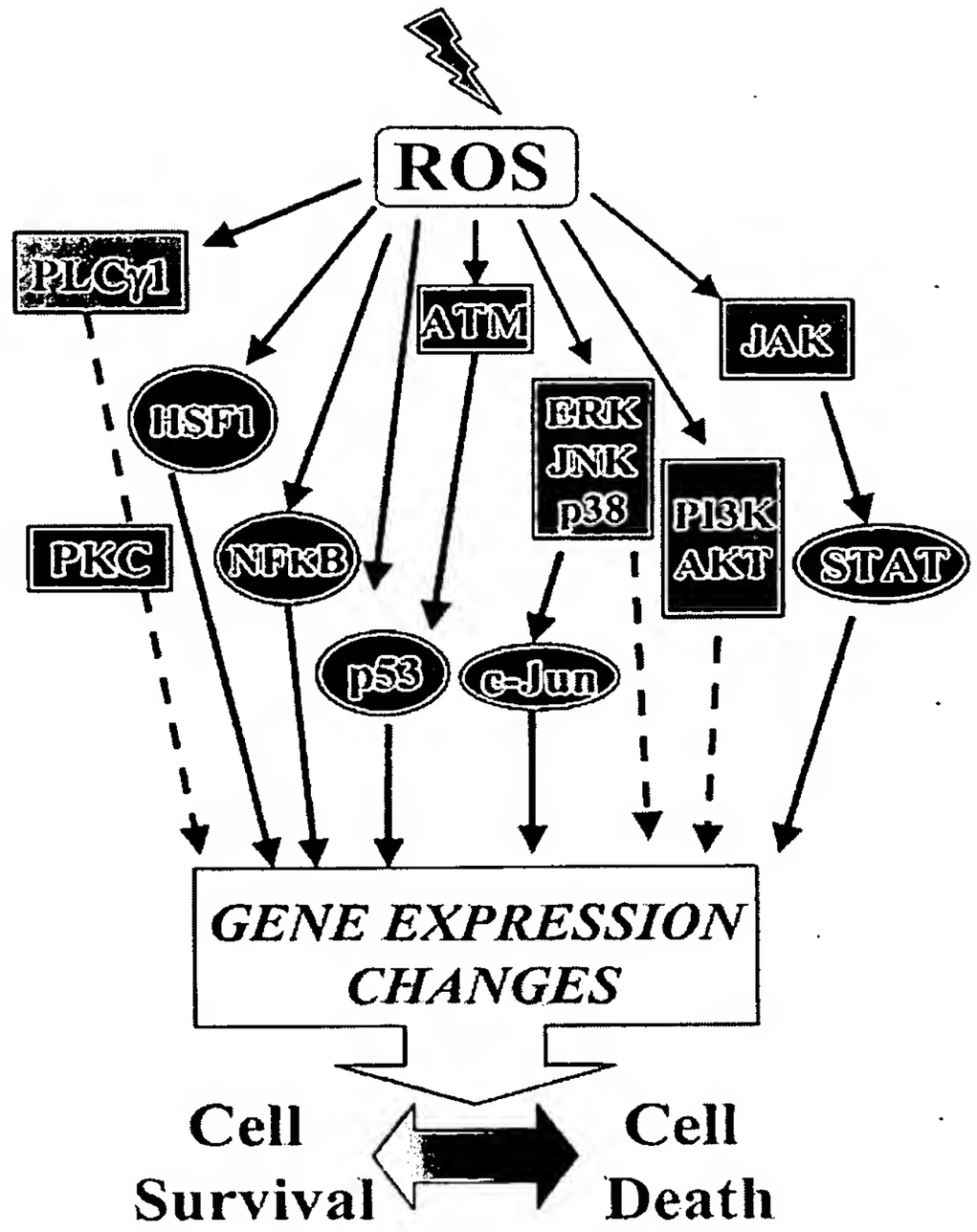

Fig. 2. Oxidative stress activates numerous major signaling pathways. Being highly reactive by nature, ROS can directly or indirectly modulate the functions of many enzymes (boxes) and transcription factors (ovals) through a multitude of signaling cascades as depicted here. Ultimately these signals result in changes in gene expression, which influence the ability of the cell to survive or die. The magnitude and duration of the stress as well as the cell type involved are important factors in determining which pathways are activated as well as the ultimate cellular outcome. ATM, ataxia-telangectasia mutated; ERK, extracellular signal-regulated kinases; HSF1, heat shock transcription factor 1; JAK, Janus protein kinase; JNK, c-Jun N-terminal kinases; NFκB, nuclear factor κB; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC-γ1, phospholipase C-γ1; STAT, signal transducers and activators of transcription.

survival. The idea that ERK and JNK act in an opposing manner to influence cell survival during stress was put forth in 1995 when it was found that growth factor withdrawal-induced apoptosis of PC12 cells was associated with decreased ERK activation and elevated JNK activation; both effects being required for the induction of apoptosis (Xia et al., 1995). This idea of opposing functions of ERK and JNK during stress was extended to oxidant injury, although in this situation ERK activity was not diminished, but, like JNK, was activated by the stress (Guyton et al., 1996a). Hence, it was suggested that the balance between the magnitude of ERK and JNK activation was key to determining survival. While this idea is still generally accepted, more recent evidence suggests that ERK can exert apoptotic influences and JNK can exert anti-apoptotic influences during the cellular response to oxidative stress. Studies examining

the role of p38 in regulating cell survival following oxidant injury have also produced conflicting results.

ERK pathway

The ERK pathway lies at the heart of many signal transduction processes and constitutes a major pathway through which growth factor receptors transduce proliferative signals to the nucleus (Chakraborti and Chakraborti, 1998; Kolch, 2000). Initially described as a pathway that was unresponsive to or downregulated by stress stimuli, it is now clear that oxidative stress leads to substantial activation of ERK and that growth factor receptors play an important role in mediating this effect. A number of growth factor receptors including the epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, and the T-cell receptor complex have been shown to undergo phosphorylation in response to oxidative insults such as hydrogen peroxide, asbestos, short wave ultraviolet light (UVC) irradiation and arsenite, and interference of these phosphorylations attenuates ERK activation in response to oxidative stress (Sachsenmaier et al., 1994; Schieven et al., 1994; Huang et al., 1996; Knebel et al., 1996; Zanella et al., 1996; Chen et al., 1998). Likewise, expression of inactive mutant forms of various growth factor receptors reduces activation of ERK by oxidative stress (Sachsenmaier et al., 1994), while overexpression of certain normal growth factor receptors, such as the Trk receptor for nerve growth factor in rat PC12 cells, results in enhanced activation of ERK by hydrogen peroxide (Guyton et al., 1998). The activation of growth factor signaling pathways by oxidants is consistent with the observation that low concentrations of hydrogen peroxide are mitogenic (Burdon, 1995). How oxidants act to initiate these events is unclear, but two possible mechanisms may be involved. First, the oxidants may actually mimic the effects of ligand-receptor interaction directly leading to activation of the growth factor receptors, perhaps through modification of cysteine residues on the receptors (Chen et al., 1998). Second, they may act to inactivate critical glutathione (GSH)-sensitive, membrane-bound phosphatases necessary for dephosphorylation (turning off) of the growth factor receptor signaling (Knebel et al., 1996; Lee et al., 1998). Recently, Meves et al. (2001) proposed that EGFR phosphorylation by hydrogen peroxide and other oxidative stress-inducing agents is preceded by GSH depletion and intracellular hydrogen peroxide accumulation.

We first reported findings implicating ERK activation as a survival factor following oxidant injury in 1996 (Guyton et al., 1996a,b). In those studies, pharmacologic agents as well as molecular alterations resulting in reduced ERK activation were found to sensitize 3T3 cells to hydrogen peroxide, while molecular strategies leading to elevated ERK activation enhanced survival of cells treated with the oxidant. Subsequent studies from a number of laboratories confirmed these findings in other cell types and with other model agents (Aikawa et al., 1997; Wang et al., 1998; Peus and Pittelkow, 2001; Ikeyama et al., 2001). However, other studies using different model systems have produced findings to suggest that ERK activation can contribute to apoptosis in response to oxidant injury. These include hyperoxia-induced apoptosis of macrophages (Petrache et al., 1999), cisplatin-induced apoptosis of HeLa cells (Wang et al., 1998), hydrogen peroxide-induced apoptosis of oligodendrocytes (Bhat and Zhang, 1999; Brand et al., 2001) and mesengial cells (Ishikawa and Kitamura, 2000), and asbestos-induced apoptosis of pleural mesothelial cells (Jimenez et al., 1997). What determines whether ERK will act in a pro-apoptotic or anti-apoptotic fashion remains an important unanswered question, but the kinetics and duration of its activation may be important factors. For example, in situations where ERK activity enhances survival, activation occurs rapidly and is more transient (Guyton et al., 1996a,b; Aikawa et al., 1997; Ikeyama et al., 2000), in situations where it is apoptotic, activation tends to be delayed and sustained (Jimenez et al., 1997; Wang et al., 2000a).

SAPK pathways

The JNK and p38 pathways are noted for their activation by a wide range of stresses including cytokines, radiation, osmotic shock, mechanical injury, heat stress, and oxidative damage. The pathways leading to their activation are extremely complex and involve multiple MAPKK, more than 10 different MAPKKK, and a variety of other interacting regulatory proteins (Kyriakis and Avruch, 2001). While some of the MAPKK and MAPKKK are selective for one pathway or the other, others are common to both JNK and p38 (Tournier et al., 2000). An important question in the field is how do such a diverse array of stresses initiate these signaling cascades? Recent studies suggest that different signals may rely on different MAPKKK and/or MAPK (Tournier et al., 2001). For oxidant-induced activation of these pathways, changes in the cellular redox state may be a key factor (Adler et al., 1999a). For example, under normal conditions the redox regulatory protein thioredoxin (Trx) has been shown to bind to and inhibit the activity of apoptosis signal-regulating kinase 1 (ASK1), a MAPKKK involved in both JNK and p38 kinase activation (Saitoh et al., 1998). However, oxidative stress causes dissociation of the Trx-ASK1 complex leading to activation of JNK and p38. CDC25A, a phosphatase that promotes cell cycle progression also binds to and inhibits ASK1 activity, and when overexpressed, CDC25A can suppress activation of p38 and JNK1 and reduce cell death triggered specifically by oxidative stress (Zou et al., 2001). Whether other MAPKKK are also subject to such redox regulation is not clear, but a similar mechanism might function at the level of JNK to influence its activity. Biochemical evidence indicates that under non-stressed conditions glutathione S-transferase (GST) binds to JNK and inhibits its activation, but that this interaction is disrupted by oxidative stress (Adler et al., 1999b). Several studies have provided evidence that JNK activation by hydrogen peroxide and/or stresses that affect the cellular redox state occurs in part through suppression of phosphatases involved in JNK inactivation (Meriin et al., 1999; Gabai et al., 2000a; Chen et al., 2001a). Thus, ROS might act at multiple levels in the JNK signaling pathway to regulate its activities. Finally, a recent study has also suggested that like ERK, JNK can be activated following hydrogen peroxide treatment via an EGFR-dependent pathway (Chen et al., 2001b).

The influence of JNK activation on cell survival following oxidative stress is also complex and highly controversial. Many studies have shown that JNK activation is correlated with cell death or apoptosis induced by agents that act at least in part via generation of ROS (see review by Chen and Tan, 2000). Some reports have been quick to conclude that JNK played a role in mediating the apoptosis, without definitive evidence. However, in such instances it is possible that JNK is activated as part of a failed attempt at survival. Given the lack of specific pharmacologic inhibitors for JNK (such as exist for the ERK and p38 kinases), most studies addressing the role of JNK in mediating apoptosis have relied on the use of dominant negative mutant forms of either JNK or its upstream activators. These approaches often yield partial effects due to the redundancy of the various signaling molecules, and therefore may either incompletely suppress JNK activation or interfere with other pathways that influence survival. Nonetheless, such strategies have supported a role for JNK in mediating apoptosis in many models of oxidative stress (Zanke et al., 1996; Turner et al., 1998; Wang et al., 1998; Yin et al., 2000). Another approach that has provided support for the role of JNK in mediating apoptosis by oxidative stress is the use of antisense oligonucleotides possessing high specificity for different JNK isoforms (Garay et al., 2000; Hreniuk et al., 2001). In these studies, JNK1 but not JNK2 has been implicated in oxidant-induced apoptosis. In keeping with the observations described above indicating that glutathione S-transferase p (GSTp) can interact with JNK to suppress its activation, it was recently shown that overexpression of GSTp likewise prevents hydrogen peroxide-induced apoptosis (Yin et al., 2000). Interestingly, in that study GSTp overexpression led to increases in ERK, p38, and IKK activities, all of which exerted protective effects against the oxidant-induced apoptosis (Yin et al., 2000). The authors have suggested a coordinate role for GSTp in regulating the response to oxidant injury. Finally, gene disruption approaches have been useful in assigning a role for JNK in mediating oxidative stress-induced apoptosis, although use of this approach has also suffered from the redundancy associated with many components of the signaling pathways and the inability to completely eliminate JNK activity. Examining the responsiveness of mouse embryo fibroblasts (MEF) from JNK1−/− and JNK2−/− mice revealed that loss of neither gene markedly affected the response to UVC irradiation. However, in MEF derived from JNK1−/− and JNK2−/− double knockouts, UVC-induced apoptosis was completely ablated (Tournier et al., 2000). ASK1 deletion, which also eliminates JNK activation in response to hydrogen peroxide treatment, renders ASK1−/− MEF resistant to apoptosis by the oxidant (Tobiume et al., 2001).

In contrast to the above findings, other studies have suggested a pro-survival function for JNK during oxidative injury. For example, deletion of another MAPKKK, MEKK1, which also inhibits JNK activation in response to hydrogen peroxide in embryonic stem cell-derived cardiac myocytes, markedly increases their sensitivity to oxidant-induced apoptosis (Minamino et al., 1999). In addition, utilizing a tetracycline-inducible system to express chimeric N-terminal JNK fragments that could block JNK substrates, Buschmann et al. (2000) found that cells expressing the N-terminal fragments were much more sensitive to hydrogen peroxide-induced apoptosis. This effect, however, relied on p53, suggesting that JNK's protective functions are circumstance-dependent. Finally, utilizing JNK antisense oligonucleotides to inhibit JNK activity, we have observed that cells are more sensitive to cisplatin-induced apoptosis, which occurs at least in part through oxidative stress (Potapova et al., 1997; Potapova, unpublished observations).

Although the p38 subfamily consists of at least five different isoforms, α, β_1, β_2, γ, and δ, most studies addressing the role of p38 in influencing cell survival following oxidant injury have focused on the α and β isoforms whose activities can be effectively inhibited by specific pharmacologic inhibitors. Such an approach has yielded evidence for both pro-apoptotic (Bulavin et al., 1999; Shimizu et al., 1999; Zhuang et al., 2000; Gratton et al., 2001) as well as anti-apoptotic (Nemoto et al., 1998; Ivanov and Ronai, 2000) functions of these kinases. In addition, although many studies have correlated p38 activation with oxidative stress-induced apoptosis, in some of these circumstances, inhibiting the kinase through use of pharmacologic inhibitors does not alter the apoptosis (Wang et al., 1998, 2000a). The influence of p38 on oxidant induced-apoptosis may also be agent-specific as recent studies by Zhuang et al. (2000) have suggested that p38 is required for apoptosis induced by singlet oxygen, but not that induced by hydrogen peroxide. Finally, it was recently shown that under conditions of low oxidative stress, where apoptosis does not occur, activation of p38 participates in mitotic arrest (Kurata, 2000).

MAPK targets and oxidative stress

Despite the numerous targets of MAPK phosphorylation that have been identified (see reviews of Chang and Karin, 2001; Kyriakis and Avruch, 2001) little is known regarding which of these are important in regulating cell survival (be it pro- or anti-apoptotic) in response to oxidative stress. c-Jun has been the most studied in this regard. Just as in the case of its upstream regulator, JNK, both pro- and anti-apoptotic functions have been ascribed to c-Jun (Bossy-Wetzel et al., 1997; Luo et al., 1998; Potapova et al., 2001), and, like JNK, c-Jun appears to function in a manner that is cell type specific, agent specific, or both. The tumor suppressor protein p53 constitutes a potential target of pro-apoptotic signaling by both JNK and p38. As will be discussed below, p53 exerts a pro-apoptotic influence in response to oxidative stress. Both JNK and p38 have been shown to be capable of phosphorylating p53, and both have been implicated in regulating p53 expression levels through stabilization of the p53 protein under conditions of stress (Fuchs et al., 1998; Bulavin et al., 1999). In the case of p38, inhibition of its activity was found to markedly reduce UVC-induced apoptosis in a p53-dependent manner (Bulavin et al., 1999). The relationship between JNK and p53-mediated apoptosis is less clear, as JNK is not required for the accumulation of p53 in response to UVC treatment (Tournier et al., 2000), nor is p53 required for JNK-induced apoptosis (Chen and Tan, 2000). In addition, c-Jun appears to exert an effect that opposes

that of JNK. That is, c-Jun leads to transcriptional repression of p53, which would be predicted to support survival. However, embryonal mouse fibroblasts lacking c-Jun display enhanced survival following UVC (Shaulian et al., 2000). It should be emphasized, however, that the above studies all centered on UVC treatment, and while oxidative stress certainly contributes to the UVC response, the possibility that the effects observed also reflect other influences of the treatment, such as direct damage to DNA, cannot be excluded.

PI3-KINASE/AKT PATHWAY

Akt, also known as protein kinase B, is a serine/threonine kinase which, like ERK, plays a key role in integrating cellular responses to growth factors and other extracellular signals (Kandel and Hay, 1999; Meier and Hemmings, 1999). Akt is activated in response to these signals via a phosphoinositide 3-kinase (PI3K) pathway in which PI3K-mediated generation of 3′-phosphorylated phosphoinositides leads to the recruitment of Akt to the cell membrane where it undergoes phosphorylation by kinases such as the 3′-phosphoinositide-dependent kinase-1 (PDK-1). Akt is an important anti-apoptotic protein through which survival signals suppress cell death induced by growth factor withdrawal, cell cycle discordance, and detachment of cells from their extracellular matrix, but its role in influencing cell fate during other conditions of stress has been more controversial. Several studies have reported that environmental stresses capable of inducing apoptosis, such as UVC, ionizing radiation (IR), and hyperosmotic stress lead to the downregulation of the PI3K/Akt pathway (Meier et al., 1998; Zhou et al., 1998; Zundel and Giaccia, 1998), and it has been suggested that such downregulation is important in the apoptotic process. However, Akt is activated in response to oxidant injury as well as several other stresses known to exert their cytotoxic effects in part through generation of ROS or perturbations in cellular redox status (Konishi et al., 1997; Sonoda et al., 1999; Klotz et al., 2000; Wang et al., 2000b; Huang et al., 2001).

Similar to that of ERK, activation of Akt in response to oxidant exposure appears to be mediated largely through growth factor receptors. For example, activation of Akt by hydrogen peroxide in HeLa cells relies on the EGF receptor, as specific inhibitors of EGF activation effectively inhibited Akt activation (Wang et al., 2000b). Peroxynitrite-induced activation of the kinase in primary fibroblasts, on the other hand, was found to rely on the PDGF receptor and was not affected by inhibitors of EGF signaling (Klotz et al., 2000). Hydrogen peroxide-induced Akt activation in B cells requires Syk, a non-receptor protein tyrosine kinase (PTK), which is known to play a critical role in B cell receptor-mediated signaling (Ding et al., 2000). Another non-receptor PTK, focal adhesion kinase, has been implicated in the activation of Akt in glioblastoma cells (Sonoda et al., 1999). Regardless of the particular PTK implicated in initiating the early events, all of the above mentioned studies demonstrated a requirement for PI3K in mediating the activation of Akt.

Evidence suggesting that activation of PI3K/Akt signaling during the cellular response to oxidant injury was important for survival was initially obtained using the PI3K inhibitor wortmannin. Treatment of cells with wortmannin blocked activation of Akt by hydrogen peroxide and increased cell death (Sonoda et al., 1999). Direct support for the importance of this pathway in enhancing cell survival following oxidant injury was subsequently obtained in studies where Akt expression and activity were modulated using a genetic approach (Wang et al., 2000b). In these studies, virus-driven elevated Akt expression was shown to enhance survival of hydrogen peroxide-treated HeLa and NIH3T3 cells. These findings have been extended to other model systems of oxidative stress including treatment with neurotoxins known to generate ROS and models of oxidative preconditioning (Alvarez-Tejado et al., 2001; Han et al., 2001; Salinas et al., 2001).

The PI3K/Akt pathway is believed to transduce its survival signals through the phosphorylation-dependent suppression of intracellular apoptotic factors such as BAD, caspase 9, forkhead transcription factor, GSK3, and IKKα (reviewed in Datta et al., 1991; Kandel and Hay, 1999). For the most part, there has been little evidence linking Akt-dependent regulation of these factors to cell survival during oxidant injury. However, Kim et al. (2001a) recently reported that ASK1 (an important upstream activator of JNK and p38 as noted above) is a target of Akt phosphorylation. Akt-mediated phosphorylation of ASK1 prevented its ability to activate JNK and its downstream target ATF2, and protected cells against hydrogen peroxide-induced apoptosis. These studies not only offer a mechanism for Akt's protective influence during oxidant injury, but point to the existence of important avenues for cross talk between the PI3K/Akt and JNK signaling pathways.

PHOSPHOLIPASE C-γ1 (PLC-γ1) SIGNALING

PLC-γ1 and PLC-γ2 are essential components of a third growth factor receptor-mediated signaling pathway that is activated in response to oxidant injury. PLC-γ1 and PLC-γ2 constitute one of three types of PLC isoenzymes (γ, β, and δ), all of which catalyze the hydrolysis of PI 4,5-P2 to inositol 1,4,5-triphosphate and diacylglycerol, which act as intracellular second messengers to provoke the mobilization of Ca^{2+} and activation of protein kinase C (PKC), respectively (Rhee and Bae, 1997; Carpenter and Ji, 1999). However, only the PLC-γ subtypes are effectors of receptor tyrosine kinases, the others being activated by a variety of other agonists including various hormones. While the PLC-γ2 isoform is expressed selectively in hematopoietic cells, the PLC-γ1 form is ubiquitously expressed. PLC-γ1 and PLC-γ2 are themselves tyrosine kinase substrates and tyrosine phosphorylation is an essential step in their activation. Present in the cytoplasm of unstimulated cells, growth factor stimulation results in the translocation of the PLC-γ1 and PLC-γ2 isoforms to the membrane allowing their interaction with and phosphorylation by receptor and non-receptor tyrosine kinases.

A number of laboratories have demonstrated that PLC-γ1 undergoes phosphorylation in response to treatment with hydrogen peroxide (Blake et al., 1993; Schieven et al., 1993; Qin et al., 1995), but until recently, the mechanisms involved in the activation process, as well as its functional significance, have been unclear. In

most studies, phosphorylation of PLC-γ1 either required or was enhanced by co-treatment of cells with vanadate, suggesting that oxidant-mediated inactivation of a phosphatase contributed to the effect. Studies using pharmacologic inhibitors of various kinases in mouse embryonic fibroblasts (MEF) have implicated both Src family tyrosine kinases and EGFR tyrosine kinase in hydrogen peroxide-stimulated PLC-γ1 phosphorylation (Wang et al., 2001). In Jurkat cells, we have likewise obtained evidence indicating that T cell receptor signaling is required for hydrogen peroxide-stimulated PLC-γ1 phosphorylation (Wang et al., unpublished observations).

Few studies have addressed the functional consequences of PLC-γ1 phosphorylation during the oxidative stress response. One study employing PC12 cells in which PLC-γ1 was overexpressed, suggested that elevated PLC-γ1 expression suppressed UVC-induced apoptosis (Lee et al., 1999). However, a second study by the same group found no protective effect of PLC-γ1 overexpression in NIH3T3 cells subjected to several different oxidative stress-inducing agents (i.e., hydrogen peroxide, tert-butylhydroperoxide, and cadmium chloride) (Lee et al., 2000a), although in that study PLC-β1 exerted a protective effect. We have observed that MEF derived from mice rendered deficient for PLC-γ1 by targeted disruption of both *plc-gamma1* alleles are much more sensitive to hydrogen peroxide treatment relative to normal fibroblasts (Wang et al., 2001). Reconstitution of PLC-γ1 protein expression in PLC-γ1-deficient MEF restores cell survival following hydrogen treatment, suggesting a protective function of PLC-γ1 activation during the cellular response to oxidative stress. Additional support for this notion has come from studies with human intestinal epithelial (Caco-2) cells, where EGF treatment protected cells against oxidants through PLC-γ-dependent signaling (Banan et al., 2001a). Further studies will be needed to address the generality of this effect for other cell types, and to identify the downstream targets involved in mediating the protective effects.

Protein kinase C (PKC)

PKC represents a family of phospholipid-dependent serine-threonine kinases involved in signaling pathways that regulate cell growth, cell death, and stress responsiveness (reviewed in Gopalakrishna and Jaken, 2000). Conventional PKC are dependent on calcium and stimulated by the second messenger diacylglycerol, a product of PLC activation (as noted above). PKC are structurally susceptible to redox regulation and various antioxidants can inhibit PKC-dependent cellular responses. Both pro-survival and apoptotic functions for PKC during oxidative stress have been described; the effect observed may depend on the specific isoforms involved. Hydrogen peroxide-induced PKC activation was shown to correlate with apoptosis in vascular smooth muscle cells, while necrosis became the predominant form of cell death when PKC was downregulated or inhibited (Li et al., 1999). Majumder et al. (2001) have recently reported that hydrogen peroxide treatment promotes phosphorylation of the PKCδ isoform as well as targeting it to the mitochondria resulting in apoptosis. While the PKCδ isoform may be associated with apoptosis induction, other isoforms have been shown to suppress apoptosis. Activation of PKC by phorbol ester protects nerve cells from oxidative glutamate toxicity by downregulating PKCδ, activating ERK and JNK, and inactivating p38 (Maher, 2001). Similarly, PKC activation was a requirement for the PLC-γ-dependent EGF protection of Caco-2 cells against oxidants discussed above (Banan et al., 2001b).

HEAT SHOCK PROTEIN (Hsp) EXPRESSION

The induction of heat shock proteins (Hsp) upon exposure to environmental insults constitutes the most ubiquitous and evolutionarily conserved stress response known to the living world. Hsp comprise of a group of related proteins classified into six major families according to their molecular weights (Hsp100, 90, 70, 60, 40, and small heat shock proteins). They function as molecular chaperones aiding in the assembly, folding, and translocation of various other proteins throughout the cells, and their induction during stress is believed to be important for preventing misfolding and aggregation, as well as for facilitating refolding and removal of damaged proteins (reviewed in Jolly and Morimoto, 2000).

The induction of Hsp in response to stress is mediated largely through transcriptional activation via heat shock transcription factor 1 (HSF1) (reviewed in Pirkkala et al., 2001). The common signal generated by various stress stimuli is likely to be protein damage, and in the case of oxidative stress, oxidative damage to proteins could be the activating signal. However, the mechanism whereby damage is sensed and the response initiated is still largely unknown. ROS have been implicated in the activation process as many oxidizing agents have been shown to result in Hsp induction, and treatment of cells with antioxidants prior to stresses such as heat shock attenuates the response (Gorman et al., 1999). Whether the antioxidants act directly at the level of HSF1 to modify its activity or simply act to prevent HSF1 activation via their ability to reduce the stress (i.e., prevent oxidative damage) is unclear.

Elevations in heat shock protein expression, particularly Hsp70 and the small heat shock proteins, have been shown to enhance survival of cells and prevent apoptosis during a wide variety of stress conditions (Jolly and Morimoto, 2000; Creagh et al., 2000a). These include direct oxidative damage (i.e., hydrogen peroxide or hypoxia reperfusion injury) as well as a variety of other stresses in which generation of ROS is implicated in cytotoxicity (i.e., chemotherapeutic agents, heat stress, cytokines) (Park et al., 1998; Preville et al., 1998; Wong et al., 1998; Chen et al., 1999; Creagh and Cotter, 1999; Komatsuda et al., 1999; Baek et al., 2000; Ding and Keller, 2001). In addition, elevated Hsp expression not only improves cell survival, but also reduces the oxidative damage to proteins, DNA, and lipids (Su et al., 1999; Yamamoto et al., 2000; Park et al., 1998). This is believed to contribute to the preconditioning effect of mild heat stress, in which exposure of cells, tissues, or organisms to a mild (non-lethal) heat stress confers greater resistance to a subsequent lethal challenge with heat or oxidant injury. Mild oxidative stress can likewise protect against a lethal challenge, and Hsp are implicated in this protection (Marini et al., 1996).

How do Hsp protect cells against oxidative damage? Many different mechanisms have been proposed. As already alluded to above, through their chaperone function, they are likely to protect proteins against denaturation or oxidative inactivation, or assist in the refolding of stress-modified proteins (Conconi et al., 1998). Perhaps also related to chaperone functions, studies of Ding and Keller (2001) have suggested that Hsp might act to preserve proteasome function that is otherwise inhibited by oxidative stress. Protective functions of the small heat shock proteins have been linked to their ability to decrease the intracellular level of ROS by modulating metabolism of glutathione to maintain it in a reduced state (Arrigo, 1998; Preville et al., 1999; Baek et al., 2000). Another way in which Hsp may act to promote survival and prevent cell death is through suppression of other apoptotic signaling pathways. Several laboratories have provided evidence indicating that Hsp70 can inhibit JNK activity and thereby inhibit JNK-mediated apoptosis (Gabai et al., 1997, 2000b; Park et al., 2001a). Two different mechanisms may contribute to this function. Sherman and colleagues have suggested that Hsp70 reduces JNK activity by increasing the rate of its dephosphorylation (Meriin et al., 1999; Volloch et al., 2000; Gabai et al., 2000a). An alternative mechanism is suggested by studies of Park et al. (2001a) who provided evidence that Hsp70 binds to JNK to prevent its phosphorylation by upstream kinases. Importantly, the chaperone function of Hsp was not required for this effect. A final way in which Hsp70 appears to promote cell survival during stress is through inhibition of the apoptotic machinery. For example, Hsp72 has been shown to both inhibit cytochrome C release and suppress processing and activity of caspases (Mosser et al., 1997, 2000; Buzzard et al., 1998; Creagh et al., 2000b). These effects are distinct from those involved in regulating JNK activity, and do rely on the chaperone function of the heat shock protein.

p53 SIGNALING

The tumor suppressor protein p53 is a universal sensor of genotoxic stress and, as a transcription factor, plays a critical role in regulating expression of genes involved in mediating growth arrest and/or cell death in response to such conditions (Burns and El Deiry, 1999; Sionov and Haupt, 1999). ROS appear to be involved at multiple levels in the p53 signaling. First, ROS are potent activators of p53 function and indeed the generation of ROS is believed to be a key factor in the activation of p53 by many chemotherapeutic agents as well as stimuli not classically considered as DNA damaging agents (e.g., cytokines). Second, ROS are generated downstream of p53 activation where they play a role in mediating apoptosis (Polyak et al., 1997; Minamino et al., 1999).

Activation of p53 following environmental insults occurs largely through posttranslational mechanisms that enhance its stability and increase its DNA binding activity. This is a complex process involving multiple phosphorylation and acetylation events (Sionov and Haupt, 1999; Colman et al., 2000). ROS contribute to p53 activation in many ways. One obvious means is through direct damage to DNA. In addition, there appears to be considerable cross talk with other signaling pathways activated by ROS, and these too appear to be contributing factors to p53 activation. One example of such cross talk is the aforementioned regulation of p53 by the p38 and JNK signaling pathways (Bulavin et al., 1999; Buschmann et al., 2000, 2001). A second example is the NFκB dependent upregulation of p53 in response to hydrogen peroxide treatment of T cells (Dumont et al., 1999). Another way in which ROS may regulate p53 activity is by modulating the redox status of a critical cysteine in the DNA-binding domain of the protein, hence affecting its DNA binding activity (reviewed in Meplan et al., 2000). Also a reflection of alterations in redox status, NADPH quinone oxidoreductase (NQO1) has been proposed to play a role in regulating p53 function by inhibiting its degradation (Asher et al., 2001).

Activation of p53 by genotoxic insults can either result in growth arrest or apoptosis. What determines this decision is unclear, but a number of factors are involved, including the cell type, the specific insult, and the magnitude and/or severity of the damage (Sionov and Haupt, 1999). Certain downstream targets of p53 activation have been identified as particularly important in mediating growth arrest, while others are more closely linked to apoptosis. Genes linked to growth arrest include the cyclin-dependent kinase inhibitor, p21(Waf1), important in mediating G1 arrest, and GADD45 and 14-3-3σ, which are important in mediating G2/M phase arrest (Taylor and Stark, 2001). p53 and its downstream target p21 are believed to play a role in hydrogen peroxide-induced growth arrest and replicative senescence (Chen et al., 2000). A large and growing number of p53 target genes have likewise been implicated in mediating its apoptotic effects. These include *Bax*, a pro-apoptotic Bcl-2 family member (Miyashita et al., 1994), several mitochondrial proteins, Noxa, (Oda et al., 2000a), p53AIP1 (p53-regulated-apoptosis-inducing protein 1) (Oda et al., 2000b), and PUMA (p53-upregulated modulator of apoptosis) (Nakano and Vousden, 2001; Yu et al., 2001), and several genes associated with death receptor-mediated apoptosis, *Fas*, Killer/DR5, and PIDD (Lin et al., 2000; Wu et al., 2000). There does not appear to be a single gene that is the principal mediator of p53-induced apoptosis, but rather the response involves the activation of several apoptotic genes (Sionov and Haupt, 1999; Ryan et al., 2000; Kannan et al., 2001). In addition, there is much overlap in the pattern of genes regulated by p53 whether it results in growth arrest or apoptosis.

The importance of p53 in mediating hydrogen peroxide-induced apoptosis has been well established. Using various genetic approaches to modulate p53 activity, it has been shown that elimination of p53 function enhances survival of hydrogen peroxide-treated cells (Yin et al., 1998; Kitamura et al., 1999; Buschmann et al., 2000). This is likely to be an important factor contributing to the chemotherapeutic resistance of certain tumors in which p53 is mutated. How does then p53 induce apoptosis? As already mentioned, several apoptosis-related proteins have been identified as targets of p53 regulation. However, there appears to be much more to the story than this. The activation of p53 itself results in the generation of ROS, suggesting that

an important consequence of oxidant-induced p53 activation is a further increase in the level of oxidative stress (Johnson et al., 1996; Polyak et al., 1997). The positive feedback loop may be important in achieving a critical threshold of ROS leading to a commitment to apoptosis. Exactly how p53 activation leads to elevations in ROS is unclear, but a likely possibility is through transcriptional modulation of genes that are involved in regulating cellular redox state (Polyak et al., 1997). A large number of genes demonstrating altered expression upon p53 expression are related to oxidative stress. One upregulated gene, designated PIG3, is involved with the perpetuation of ROS associated with cell death, although PIG3 overexpression itself is not sufficient to induce apoptosis (Flatt et al., 2000). Manganese superoxide dismutase (MnSOD or SOD2), a critical enzyme involved in radical scavenging, is a target of transcriptional repression by p53 (Drane et al., 2001). Indeed, forced expression of MnSOD was found to enhance resistance to p53-mediated apoptosis (Pani et al., 2000; Drane et al., 2001). Interestingly, the studies by Drane et al. (2001) suggest that MnSOD may have a reciprocal effect on p53, downregulating its transcription. Glutathione peroxidase (GPX), another gene important in regulating redox status and protecting cells against oxidative stress, is transcriptionally activated by p53 (Tan et al., 1999). Although this seems paradoxical given the ROS-generating effects of p53, the induction of GPX is an early event following p53 activation (Tan et al., 1999) while p53-mediated ROS generation occurs much later (Polyak et al., 1997). Taken together, the existing data suggest that p53 plays a role in regulating cellular redox state. Small shifts in the redox state exist and that may influence the downstream biological responses of p53 depending on the cell type, the extent of the damage and/or the duration of the stress.

p53 can also interfere with other survival signals to render cells permissive to apoptosis. p53 represses expression of Bcl-2, a known survival signal, while also increasing expression of the pro-apoptotic Bcl-2 family member Bax (Miyashita et al., 1994). In response to hydrogen peroxide, p53 also induces expression of p85, the regulatory subunit of PI3K which is associated with apoptotic activity (Yin et al., 1998). Normally, the p85 subunit links the signals from activated insulin-like growth factor-1 (IGF1) receptors to Akt through PI3K, an effect usually associated with cell survival (see above). However, increased p85 expression in the absence of receptor coupling may have a dominant negative effect on the signaling cascade from the IGF1 receptor and thus attenuate its survival influence (reviewed in Colman et al., 2000). Furthermore, p53 can downregulate expression of the survival signal IGF-II, while increasing IGF-binding protein 3 (IGFBP3) levels (reviewed in Grimberg, 2000). IGFBP3 has been shown to inhibit IGF action by competitively binding IGFs and preventing their binding to and activation of the IGF1 receptor. IGFBP3 can also act independently of IGF1 to inhibit cell growth and enhance apoptosis (see Grimberg, 2000). Finally, it has been shown that IGFBP3 can enhance and prolong p53 induction in response to UVC-radiation, thereby significantly enhancing apoptosis (Hollowood et al., 2000).

Despite the predominance of its pro-apoptotic effects during oxidative stress, p53 activation can activate other pathways and genes that may be involved in a compensatory mechanism to alleviate adverse effects of oxidative stress. As already noted, *GPX* is one such gene belonging to this category. Another gene in this group, heparin-binding epidermal growth factor-like factor (HB-EGF), was recently identified as upregulated by p53 in response to DNA damage. In a manner similar to that of EGF, it was found to activate pathways leading to ERK and Akt activation, and thereby protect cells from cytotoxicity following subsequent treatment with hydrogen peroxide (Lee et al., 2000b).

Ataxia-telangiectasia mutated (ATM) kinase

Optimal induction and activation of p53 protein, after ionizing radiation (IR) and certain other forms of oxidative stress, require the activation of the ataxia-telangiectasia mutated (ATM) kinase, a member of the PI3K-like family of kinases which also include DNA-PK (Banin et al., 1998; Canman et al., 1998). Based on our previous discussion, it might be predicted that cells deficient in ATM function would show reduced sensitivity to oxidative insults. This, however, is not the case, as patients with AT exhibit hypersensitivity to IR and a reduced ability to respond appropriately to oxidative challenge (Lavin and Shiloh, 1997; Gatei et al., 2001). In response to oxidative challenges or DNA damage, AT cells show reduced induction of p53 protein and p53 phosphorylation, and they fail to show G1/S and G2/M cell cycle checkpoints as compared to cells with wild type ATM (Shackelford et al., 2001). It is the loss of these critical p53-dependent checkpoint functions that is believed to render AT cells hypersensitive to IR. A recent report showed that the hydrogen peroxide-induced phosphorylation of p53 on multiple serine residues was blocked in ATM−/− cells (Xie et al., 2001). Specifically, serine-20 of p53 was phosphorylated by polo-like kinase-3 (Plk3) which demonstrated hydrogen peroxide-induced activity dependent also on ATM function. Serine-20 phosphorylation is important for p53 stabilization because this modification interferes with p53's association with its inhibitor, Mdm2, a primary mediator of p53 degradation (Fang et al., 2000). Chk2, another kinase capable of phosphorylating serine-20 of p53 (Hirao et al., 2000), is directly phosphorylated by ATM following IR and by ATR (ATM and Rad3-related) following UVC treatment (Matsuoka et al., 2000; reviewed in Shiloh, 2001). Thus ATM directly and indirectly mediates many p53 modifications that will stabilize and activate the protein.

It has been hypothesized that ATM somehow acts as a sensor of ROS and/or oxidative damage (Rotman and Shiloh, 1997) and perhaps is involved in a specialized antioxidant function to maintain cellular homeostasis such that its absence or dysfunction in AT cells renders them less capable of dealing with oxidative stress (Formichi et al., 2000; Takao et al., 2000). Furthermore, it has been suggested that the absence of functional ATM results in a mild and continuous state of oxidative stress (Barlow et al., 1991; reviewed in Rotman and Shiloh, 1997; Gatei et al., 2001; Kamsler et al., 2001). While the mechanisms remain unclear, the existing data suggest that ATM serves protective roles

in response to oxidative stress via signaling to downstream effectors of the cell cycle checkpoint functions thus allowing for repair of oxidative damage.

NUCLEAR FACTOR κB (NF κB) SIGNALING

The NFκB family of transcription factors is composed of homodimers or heterodimers of Rel proteins that are involved in regulating a large number of genes related to immune function, inflammation, apoptosis, and cell proliferation (Sha, 1998; Pahl, 1999). Since many of the treatments known to activate the transcription factor also lead to ROS production (e.g., cytokines and radiation), and antioxidants can effectively block NFκB activation in response to such stimuli, ROS were once implicated as central mediators in the NFκB activation process (Schreck et al., 1991; Schmidt et al., 1995, 1996). However, more recent studies have failed to provide support for this hypothesis as reviewed in Li and Karin (1999) and Bowie and O'Neill (2000). Most important among these studies are the findings that (1) activation of NFκB in response to hydrogen peroxide is cell type-specific; (2) an increase in ROS is not required for NFκB activation in many instances; and (3) antioxidants may inhibit NFκB activity through mechanisms distinct from redox regulation. Nonetheless, in certain cell types, oxidative stress is a potent activator of NFκB and this can have important consequences for cell survival.

How do ROS activate NFκB? The predominant mechanism by which NFκB is activated by various stimuli is through the phosphorylation of IκB. IκB is an inhibitory protein that under normal conditions binds to NFκB and sequesters it in the cytoplasm, thereby preventing its access to DNA. The phosphorylation of IκB results in its ubiquitination and degradation, freeing NFκB to translocate to the nucleus and activate transcription through sequence-specific binding to DNA. For most inducers of NFκB activation, IκB phosphorylation occurs on serine residues S32 and S36, and a number of different kinases have been reported to phosphorylate IκB on these sites. These include Iκ-kinase (IKK), NFκB-inducing kinase (NIK), double-stranded RNA-activated serine-threonine protein kinase (PKR) (reviewed in Schoonbroodt and Piette, 2000; Janssen-Heininger et al., 2000), p90RSK (Ghoda et al., 1997), MEKK1 (Hirano et al., 1996; Meyer et al., 1996), and Akt (Madrid et al., 2001). Many of these kinases offer obvious points for cross talk with other signaling pathways known to be activated by oxidant injury. A recent report found that hydrogen peroxide-induced IL-6 promoter activity and gene expression by NFκB activation was mediated by peroxide-induced NIK autophosphorylation that increased IκB degradation (Zhang et al., 2001). Other evidence suggests, however, that hydrogen peroxide and hypoxia/reoxygenation can lead to phosphorylation of IκBα on an alternative site from that noted above. This phosphorylation occurs on tyrosine-42 within the PEST domain on the C-terminal region of IκBα and leads to the displacement of IκBα from NFκB and subsequent digestion by calpain proteases (Schoonbroodt et al., 2000). In Jurkat cells, tyrosine-42 phosphorylation of IκBα involves both p56Lck and ZAP-70 (Livolsi et al., 2001). The tyrosine-42 residue lies within a consensus sequence for binding the regulatory subunit of PI3K, p85, and tyrosine-42-phosphorylated IκB has been shown to stably interact with p85 following pervanadate treatment of Jurkat cells (Imbert et al., 1996; Beraud et al., 1999).

Virtually every step of the NFκB signaling cascade is comprised of redox-sensitive proteins whose activities are modulated upon changes in ROS, some of these in a negative fashion (reviewed in Flohe et al., 1997; Janssen-Heininger et al., 2000; Schoonbroodt and Piette, 2000). NFκB must be in a reduced form to exhibit DNA binding activity, thus reducing agents (dithiothreitol and mercaptoethanol) enhance DNA binding activity, while oxidizing agents (diamide) inhibit this activity. A two-step mechanism of redox regulation has been proposed as thioredoxin, a cellular reducing catalyst can block degradation of IκB in the cytoplasm and inhibit NFκB activation, but can enhance NFκB transcriptional activities by enhancing its ability to bind DNA when present in the nucleus (Hirota et al., 1999). A large number of NFκB-dependent genes have anti-apoptotic functions. These include TNF receptor-associated factor 1 (TRAF1), TRAF2, cellular inhibitors of apoptosis proteins (CIAPs), MnSOD, and A20 zinc finger protein (reviewed in Pahl, 1999; Bours et al., 2000). NFκB is also involved in regulating the expression of Bfl-1/A1 and Bcl-XL, two anti-apoptotic members of the Bcl2 family. Accordingly, NFκB expression has been shown to exert protective effects under various conditions. However, NFκB activity has also been correlated with apoptosis and the activation of apoptosis-associated genes such as *Fas* ligand and *p53* (Wu and Lozano, 1994; Matsui et al., 1998; Kasibhatla et al., 1999). Studies examining the influence of NFκB on cell survival following oxidant injury have likewise produced mixed findings. Although some studies have provided evidence for a protective function of NFκB in response to oxidative stress (Mattson et al., 1997, 2000; Yu et al., 2000; Kim et al., 2001b) more studies support the notion that NFκB exerts a pro-apoptotic effect following oxidant injury (Luo et al., 1999; Vollgraf et al., 1999; Shou et al., 2000; Aoki et al., 2001). In addition, the finding that p53-mediated cell death depends on NFκB supports such a pro-apoptotic function (Ryan et al., 2000). Finally, it is worth noting that there are cases in which NFκB has been shown to be activated in response to oxidant injury, but with no apparent influence on survival (Wang et al., 1998).

OTHER SIGNALING MOLECULES AND PATHWAYS

JAK/STAT pathway

Cytoplasmic Janus protein tyrosine kinases (JAKs) are critical components of multiple signaling pathways that govern survival, proliferation, and apoptosis (reviewed in Rane and Reddy, 2000). The primary substrates for JAK kinases are cytokine receptors which dimerize or oligomerize upon ligand binding, but JAK kinases are also known to activate members of the signal transducers and activators of transcription (STAT) family. Once the STAT factors are tyrosine phosphorylated by JAKs, they can dimerize and translocate to the nucleus where they have been shown to activate or repress transcription (Rane and Reddy, 2000).

The JAK-STAT pathway is activated in response to oxidative stress as demonstrated by the activation of the STAT factors STAT1 and STAT3 and the STAT kinases JAK2 and TYK2 by hydrogen peroxide in the absence of new protein synthesis (Simon et al., 1998). This activation can be inhibited by antioxidants, but is somewhat oxidant-specific in that neither superoxide nor nitric oxide could activate the STATs. Carballo et al. (1999) suggest that the hydroxyl radical might activate STAT3 perhaps through the inhibition of an intracellular tyrosine phosphatase important for its dephosphorylation.

As far as targets of the JAK/STAT pathway that might influence the cellular outcome following oxidative stress, few genes have yet been identified. Recent studies have indicated that activation of Hsp70 by hydrogen peroxide occurs at least in part via enhanced binding of STATs to cognate binding sites within the Hsp70 promoter (Madamanchi et al., 2001). Inhibition of JAK2 activity partially inhibited the hydrogen peroxide-induced Hsp70 expression (Madamanchi et al., 2001). Also, the promoter of the human heme oxygenase gene which encodes a stress protein that participates in the defense mechanisms against oxidative injury, has a putative STAT3 binding site that contributes to its induction by IL-6 (Deramaudt et al., 1999). Finally, JAK2/3 seem to be involved in the enhanced connective tissue growth factor expression observed after hydrogen peroxide treatment (Park et al., 2001b). These studies suggest that activation of the JAK/STAT signaling cascades assists the cell in surviving oxidative stress situations.

c-Abl tyrosine kinase

c-Abl is a non-receptor tyrosine kinase that is distributed in the nucleus and the cytoplasm of proliferating cells. Both forms of the c-Abl are activated by genotoxic stresses, including oxidative damage (Sun et al., 2000a,b), with activation being associated with cell death. In the case of hydrogen peroxide, PKCδ (Sun et al., 2000b) is believed to mediate activation of nuclear c-Abl, targeting it to the mitochondria where it takes part in initiating apoptosis (Kumar et al., 2001). Accordingly, cells deficient in c-Abl show attenuated cytochrome C release and reduced apoptosis in response to hydrogen peroxide treatment (Sun et al., 2000a).

There is also evidence to suggest that c-Abl might enhance apoptosis in response to oxidative stress through modulation or interference of other signaling pathways. For example, it has been shown to suppress activation of Akt, while enhancing activation of JNK and p38 through its activation of upstream MAPKKs MEKK1 and MKK6 (Cong and Goff, 1999; Kharbanda et al., 2000). However, the effects appear to be rather specific for certain types of genotoxic stress, and thus the relevance to oxidative stress per se remains unclear.

$p66^{shc}$ adapter protein

$p66^{shc}$ is one of three splice variants encoded by the Shc adapter protein gene. The $p52^{shc}$ and $p46^{shc}$ adapter proteins play important roles as transducers of mitogenic signals from activated receptors to Ras, but the function of $p66^{shc}$ in this regard is less clear (Migliaccio et al., 1997). The $p66^{shc}$ protein is unique among the Shc isoforms in that it shows marked and sustained phosphorylation on serine residues during the stress response to UVC or hydrogen peroxide (Migliaccio et al., 1999). MEF rendered null for $p66^{shc}$ expression display enhanced resistance to hydrogen peroxide-induced apoptosis and impaired p53-dependent apoptotic pathways. Reconstitution of wild type $p66^{shc}$ expression in these cells increases their sensitivity to hydrogen peroxide, while cells expressing mutant forms of the protein that cannot be serine phosphorylated behave like null cells (Migliaccio et al., 1999). In a different model system, Andoh et al. (2000) showed that the downregulation of $p66^{shc}$ caused by serum starvation and concomitant with an upregulation of the anti-apoptotic Bcl-2 was associated with enhanced tolerance to oxidative challenges. Taken together, these data suggest that $p66^{shc}$ is a crucial component of the apoptotic response to oxidative damage (reviewed in Lithgow and Andersen, 2000; Skulachev, 2000).

SUMMARY AND CONCLUSIONS

The past few years have witnessed a tremendous growth in our knowledge concerning the signal transduction pathways involved in regulating cellular responses to stress, and of particular interest in this review, those important in mediating response to oxidative stress. With this has come also an appreciation for the complexity of the response and awareness that the individual signaling pathways do not act in isolation, but rather can intersect with and modulate one another's activities. Thus, the combined effects of individual pathways can be either additive or antagonistic, and depend not only on the oxidative agent examined, but also the dose employed and the cellular context in which they are analyzed. Despite these many caveats, certain generalities have emerged from the data regarding the individual pathways and their roles in influencing survival. As summarized in Table 1, certain pathways clearly tend to favor survival, while others promote cell death. Hence, the final outcome reflects the relative balance between the activities activated in a given cell. Whether survival or cell death

TABLE 1. Signaling pathways activated by oxidative stress and their ultimate cellular outcomes

	Cellular outcome	
Signaling pathways	Enhanced survival	Cell death
p53	+	+++
NFκB	+	+++
HSF1	+++	–
PI3K/Akt	+++	–
ERK	+++	++
JNK	++	+++
p38	+	+
PLCγ	+++	–
JAK/STAT	+++	–
c-Abl	+	+++

This table summarizes the prevailing evidence regarding the activation of these major pathways and how their downstream targets either enhance cell survival or promote cell death specifically in response to oxidant injury. (–), there is minimal or no evidence that this pathway influences this outcome. (+), indicates the degree to which this outcome appears to be affected by this pathway: +, some evidence for this outcome; ++, much evidence that this pathway promotes this outcome; +++, predominant outcome for this pathway.

is the desired response will also vary with the particular circumstances or disease condition. For instance, in the case of cancer treatment and/or prevention, apoptosis may be the desired response, while for neuro-degenerative diseases improved cell survival would more likely be the favored outcome. Whatever the goal, modulation of pathways involved in mediating cellular responses to oxidant injury offer unique opportunities for therapeutic interventions aimed at treatment of diseases or conditions, such as normal aging, where oxidative stress is an important factor.

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